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NEUROTOXICOLOGICAL EFFECTS OF CHLORPYRIFOS AND DELTAMETHRIN ON DOPAMINE AND ACETYLCHOLINE SYSTEMS IN DROSOPHILA MELANOGASTER

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Hala Husam Abdul Baki

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Dr. Mohammad Ali Al-Deeb

November 2021

Declaration of Original Work

I, Hala Husam Abdul Baki, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Neurotoxicological Effects of Chlorpyrifos and Deltamethrin on Dopamine and Acetylcholine Systems in Drosophila Melanogaster*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Mohammad Ali Al-Deeb, in the College of Science at UAEU. This work has not previously been presented or published or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Emerging evidence suggests a positive association between pesticide exposure and sporadic Parkinson's disease (PD) development. The molecular mechanisms of PD and other neurodegenerative diseases are not fully understood, which hinders the development of therapeutic agents to cure or prevent the development of such diseases. *Drosophila* has been widely used as a model organism to study various neurodegenerative diseases and to screen for promising therapeutic agents. The aims of this study were: (i) investigating the toxic effect of 24 hours exposure to chlorpyrifos (CPF) and deltamethrin (DLM) on the dopaminergic system and AChE activity in *Drosophila melanogaster*; (ii) comparing different methodologies to test negative geotaxis behavior in *D. melanogaster*; and (iii) investigating the combined neuroprotective effects of ferulic acid (FA), and Thymoquinone (TQ) natural compounds on DLM induced neurotoxicity. Adult male wild-type flies were exposed to either CPF, DLM, for 24 hours, or concomitantly exposed to DLM and individual neuroprotective agents, or their mix for 72 hours in 10% sucrose on a cotton swap. CPF/DLM-treated flies climbed shorter distances in the negative geotaxis assay as well as had a higher incidence of mortality when compared to the control group. Evidently, CPF/DLM exposure caused a disturbance in the expression of DA-related genes. The DLM exposure for 72 hours caused a higher incidence of mortality and severe locomotor defects. Co-treatment with neuroprotective agents offered protection against these toxic effects of DLM after 72 hours. DLM caused significant inhibition of AChE which was ameliorated with the concomitant exposure with FA. Our results suggest that FA and TQ were effective in reducing the toxicity induced by DLM in *D. melanogaster* as well as confirm the utility of this model to investigate potential

therapeutic strategies on movement disorders, including PD. The present study indicates that a single molecule can interact and affect multiple systems that are not related to their main mechanism of action. Data gathered in the present study may be important for the assessment of the safety of insecticides that humans are at risk of daily exposure to them. Moreover, this study presents a potential natural compound that can ameliorate and protect against the neurotoxicity that is caused by these insecticides.

Keywords: Sporadic Parkinson's disease, Neuroprotective agents, Pesticides, *Drosophila melanogaster*.

Title and Abstract (in Arabic)

التأثيرات السمية العصبية للكلوربيريفوس والديلتاميثرين على أنظمة الدوبامين والأسيتيل كولين في ذبابة الفاكهة

الملخص

تشير الأدلة الناشئة إلى وجود ارتباط إيجابي بين التعرض للمبيدات ونشوء مرض الشلل الرعاشي مجهول الأسباب. الآليات الجزيئية لمرض الشلل الرعاشي والأمراض التنكسية العصبية الأخرى غير مفهومة تمامًا، مما يعيق تطور العوامل العلاجية لعلاج أو منع تطور هذه الأمراض. تم استخدام ذبابة الفاكهة، (*Drosophila melanogaster*) على نطاق واسع كنموذج لدراسة مختلف الأمراض التنكسية العصبية، (neurodegenerative diseases) وللتحقق من العوامل العلاجية الواعدة. تهدف هذه الدراسة إلى : (1) البحث في التأثير السام بعد 24 ساعة من التعرض للمبيدين: الكلوربايريفوس و الدلتاميثرين على نظام الدوبامين ونشاط الأسيتيل كولين في ذبابة الفاكهة؛ (2) مقارنة عدة طرق مختلفة لاختبار السلوك الحركي لذبابة الفاكهة؛ و (3) البحث في التأثيرات الإيجابية لحمض الفيروليك (Ferulic acid) (FA) و الثايموكوينون (TQ) (Thymoquinone)، أو مزيجهما للحماية ضد التأثيرات السمية العصبية الناتجة عن التعرض للديلتاميثرين لمدة 72 ساعة. ذباب الفاكهة الذي تعرض للكلوربايريفوس أو الديلتاميثرين أدى أسوأ عند قياس السلوك الحركي و إلى ارتفاع معدل الوفيات عند مقارنته بالمجموعة الغير معالجة بالمبيد. في الواقع ، تسبب التعرض للكلوربايريفوس أو الديلتاميثرين في اضطراب في الجينات المرتبطة بنظام الدوبامين .تسبب التعرض لـلديلتاميثرين لمدة 72 ساعة في ارتفاع معدل الوفيات ونقص السلوك الحركي. قدم العلاج المشترك مع عوامل الحماية العصبية الحماية ضد هذا التأثير السام لـلديلتاميثرين بعد 72 ساعة. تسبب الديلتاميثرين في تثبيط كبير للـ (AChE) و الذي تم

استعادة جزء من نشاطه مع التعرض المصاحب لحمض الفيوليك. تشير نتائجنا إلى أن FA و TQ كانا فعالين في الحد من السمية التي يسببها الديلتاميثرين في ذبابة الفاكهة بالإضافة إلى تأكيد فائدة ذبابة الفاكهة في ايجاد مركبات ذات قيمة علاجية ضد اضطرابات الحركة, بما في ذلك مرض الشلل الرعاشي. تشير الدراسة الحالية إلى أن المبيد الواحد يمكن أن يتفاعل ويؤثر على أنظمة متعددة لا تتعلق بآلية عملها الرئيسية. قد تكون البيانات التي تم جمعها في الدراسة الحالية مهمة لتقييم سلامة المبيدات الحشرية التي نتعرض لها بشكل يومي. علاوة على ذلك ، تقدم هذه الدراسة مركبًا طبيعيًا محتملًا يمكن أن يحمي ضد السمية العصبية التي تسببها هذه المبيدات الحشرية.

مفاهيم البحث الرئيسية: مرض الشلل الرعاشي ، عوامل الحماية العصبية، المبيدات الحشرية، ذبابة الفاكهة.

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Dedication

I would like to dedicate my work to my beloved parents and family

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List of Abbreviations

AChE	Acetylcholinesterase
ACh	Acetylcholine
AD	Alzheimer's' Disease
Aanat1	Arylalkylamine N-Acetyltransferase1
cAMP	Cyclic Adenosine Monophosphate
CNS	Central Nervous System
COMT	Catechol-O-Methyltransferase
CPF	Chlorpyrifos
DA	Dopamine
Dat	DA Transporter
Ddc	Dopa-Decarboxylase
DLM	Deltamethrin
D. melanogaster	Drosophila Melanogaster
Dopa	Dihydroxyphenylalanine
Dop1r1	Dopamine Receptor 1
Dop2r	Dopamine Receptor 2
Dopecr	DA/Ecdysteroid receptor
ECVAM	European Centre for the Validation of Alternative Methods
FA	Ferulic Acid
LC50	The median-lethal concentration
MAO	Monoamine Oxidase
ND	Neurodegenerative Diseases/Disorders
OPs	Organophosphates
PD	Parkinson's Disease
Ple	Tyrosine Hydroxylase (Drosophila)

RING	Rapid Iterative Negative Geotaxis
ROS	Reactive Oxygen Species
RT-qPCR	Quantitative Real-Time Polymerase Chain Reaction
SNpc	Substantia Nigra Pars Compacta
TH	Tyrosine Hydroxylase
TQ	Thymoquinone

Chapter 1: Introduction

1.1 Overview

Pesticides have been proven to disrupt the balance of the ecosystem. When pesticides are used, they will also kill non-pest organisms. Although the use of pesticides is beneficial, there are also many problems related to their use. When pesticides are used, they do not always stay where they are used. They are mobile in the environment and often move in water, air, and soil. The problem with pesticide mobility is that when traveling, pesticides may come into contact with other organisms and cause damage. Another major issue related to pesticide use is bioaccumulation, where the accumulation of a substance in the body occurs because the body does not have the proper mechanism to remove it. Many synthetic pesticides cannot be decomposed. Once they enter the organism, they are permanently stored in the body tissues. After countless studies, pesticides have been linked to cancer, AD, and even birth defects. Pesticides can also damage the nervous, reproductive, and endocrine systems. Pesticides are very harmful even to the fetus because mothers release these chemicals during pregnancy or while breastfeeding their children. Although a slice of fruit containing pesticides will not kill humans, however, once they accumulate in the body, they may be harmful and should be avoided as much as possible.

At present, the molecular mechanisms of PD and other neurodegenerative diseases are not fully understood, which hinders the development of therapeutic agents to cure or prevent the development of such diseases. These facts highlight the importance of model organisms like *Drosophila* for understanding the molecular mechanisms of Parkinson's disease.

As natural compounds have shown promise for treating PD. Therefore, these compounds can lay the foundation for new therapies to treat these diseases. To date, there are no therapeutic options that can counteract the progression of NDs. Natural products are usually able to modulate the progression of these diseases, which is evident in their reduction of oxidative stress.

1.2 Statement of the Problem

Many recent studies have found that pesticides have negative neurological effects in mammals, and epidemiological studies have found a link between environmental pesticide exposure and sporadic PD. Adding to that the general population is readily exposed, either through the ingestion of pesticides treated food or through indirect exposure. It is important to understand the mechanism by which these pesticides are causing damage to the nervous system by study their effect on the molecular levels. And since up to the current date, there are no established curative or preventative interventions for managing NDs such as PD. Consequently, screening for natural antioxidants that can act as a disease-modifying agent or at least that can delay the disease progression is crucial. Of course, plant-derived compounds have become leaders in drug research. The mechanism of action of many natural antioxidants is still unknown, which hinders drug development. considering the positive interactions of different natural compounds is important. *In vivo* studies using model organisms can provide valuable insights into the neuroprotection role of these natural compounds. Thus the aims of this study are to (i) investigate the toxic effect of 24 hrs exposure to CPF on the dopaminergic system and AChE activity in *D. melanogaster*; (ii) compare different methodologies to test negative geotaxis behavior in *D. melanogaster*; (iii) investigate the toxic effect of 24 hrs exposure to DLM on the dopaminergic system

and AChE activity in *D. melanogaster*; and (iv) test the combined neuroprotective effects of FA, and TQ natural compounds on DLM induced neurotoxicity.

1.3 Pesticides

Pesticides are chemical compounds used to control plants, molds, and insects. They are classified according to the pests they control. Insecticides, herbicides, and fungicides are examples of pesticide subcategories.

Pesticides are playing an important role in agriculture by increasing food production and public health by reducing the probability of getting a vector-borne disease such as dengue fever, malaria, and schistosomiasis. (Mossa et al., 2018; Wilson et al., 2020). These pesticides are likewise utilized for different purposes, such as nonagricultural uses that include, but are not limited to, gardens, roadsides, golf courses, and pet shampoos (Hoffman et al., 2000).

1.3.1 Neurotoxicity of Pesticides

Pesticides have shown undesirable harmful effects on non-target organisms (e.g., humans/ wildlife populations), because of their intrinsic toxicity and limited species selectivity. unexpectedly, humans will be affected by these compounds. Although, they are already used in small amounts and humans are much larger than the target species for pesticides. However, these compounds are indeed causing a harmful toxic effect on humans which is not only associated with high doses, responsible for acute poisonings, but even with low doses, as in case of being exposed to mixtures of pesticides (Hernández et al., 2013), or as in case of chronic exposure where long-term exposures may affect the human health by increasing the incidence of cancer, certain neurological disorders, respiratory disorders (Bassil et al., 2007;

Hernández et al., 2013; Kim et al., 2017; Parrón et al., 2011) at the level of the general population. Many factors can determine the possible health outcome including the type of pesticide, the duration, route of exposure, and the individual health status (e.g., damaged skin/ nutritional deficiencies) (Kim et al., 2017). Pesticides might be metabolized then excreted, or bioaccumulated within a human or animal body (Alewu & Nosiri, 2011; Mesnage et al., 2018). Adding that the general population is readily exposed, either through the ingestion of pesticides treated food or through indirect exposure.

Insecticides are of two types; the first is synthetic insecticides which are assigned into different groups based on their chemical structures and mode of toxicity, such groups are: organochlorines, organophosphates, carbamates, and pyrethroids (Meijer et al., 2014; Mossa et al., 2018). Previous studies demonstrated that synthetic insecticides such as paraquat, maneb, dieldrin, pyrethroids, and organophosphates share the ability to cause oxidative stress, mitochondrial dysfunction, α-synuclein fibrillization, and neuronal cell loss in experimental animals (Baltazar et al., 2014). Long-term usage of synthetic pesticides has resulted in residues accumulating in food, water, soil, and other environmental components, where they can have negative health consequences for humans and ecosystems.

The second type is natural insecticides such as azadirachtin, rotenone, and spinosad. Natural insecticides are not completely safe, and some natural substances are poisonous; arsenic and nicotine, for example, have been employed as natural pesticides in the past. Currently, these natural compounds are not used as pesticides as they are considered unsafe. Others can even induce adverse effects in experimental animals via the induction of oxidative stress, such toxicities are renal toxicity,

hepatotoxicity, neurotoxicity, and reproductive toxicity. They can also induce genotoxicity, mutagenicity, and carcinogenicity in mammals (Mossa et al., 2018).

In recent years, epidemiological and toxicological studies have shown that insecticides act as toxins and can cause neuronal degeneration and other pathogenesis (Ghosh et al., 2014).

1.3.2 Organophosphates

The organophosphate (OP) compounds are synthetic organic chemicals that were used as pesticides and chemical warfare agents shortly after their creation due to their acute and potent neurotoxic effects (Balali-Mood, 2013). Pesticides, warfare agents, ophthalmic products, plasticizers, and other OP ester-derived products exploded in popularity in the 20th century, increasing their release to the environment and human exposure to OP compounds through a variety of routes (Terry Jr, 2012; Balali-Mood, 2013). Despite that the first reported toxicological effects described only cholinergic symptoms caused by acute exposure to OPs, however, many toxicological effects, including genotoxicity, decreased fertility, hepatotoxicity, and cancer development (Kwong, 2002; Sanchez-Pena et al., 2004; Binukumar et al., 2010; Band et al., 2011), now appear to be linked to chronic and low-dose exposures, even at doses with no observable cholinergic etiology. These findings lead to the hypothesis that OP chemicals interact with a variety of other molecular targets and have a variety of consequences, which could explain why some diseases arise after exposure. organophosphates can lead to neurotoxicity via multiple mechanisms. Recent data have implicated oxidative stress in both acute high-level and repeated low-level OP exposure.

Organophosphate compounds are phosphoric acid esters that are widely used as insecticides. Their oxone metabolites inhibit acetylcholinesterase (AChE), thereby slowing down the breakdown of acetylcholine (Ach) and stimulating the cholinergic system in the brain (Figure 1).

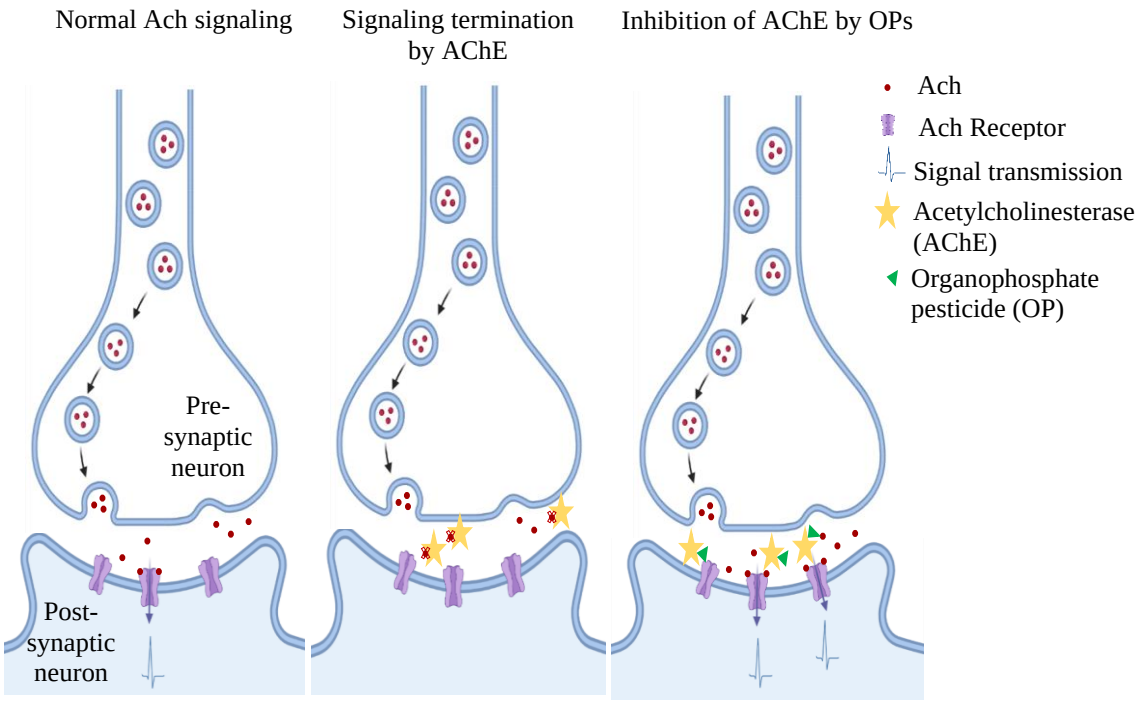

Figure 1: The mechanism of action of organophosphate pesticides. This figure was reproduced from (washington.edu).

Undesirable neurotoxicity can occur even at doses lower than the one that inhibits AChE, (Slotkin et al., 2006; Turton et al., 2021). Extremely strong OPs were used as nerve gas (such as sarin, soman, tabun, and VX). The milder OPs are often used as insecticides. Commonly used OP insecticides include parathion, methyl parathion, malathion, dichlorvos, and chlorpyrifos (CPF). Parathion and methyl parathion are completely banned in the US, but the rest are still widely used in households, agriculture, and other landscapes like golf courses. (Rush et al., 2010).

1.3.3 Chlorpyrifos

Chlorpyrifos (Figure 2) is an organophosphate insecticide, largely used for controlling pests in crops due to its lower persistence in the environment. In the last decades, the expansion of agricultural practices has led to the indiscriminate deposition of xenobiotics such as organophosphate pesticides in ecosystems which causes damage both to the environment and to human health (Soltaninejad & Shadnia, 2014).

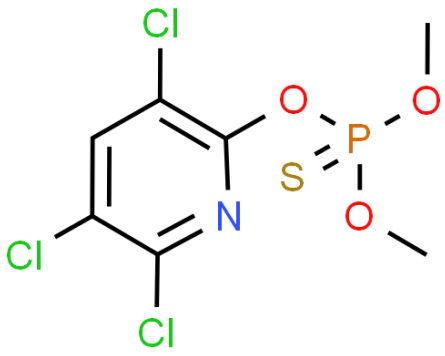

Figure 2: The chemical structure of CPF (chemspider.com)

The main target for CPF is the AChE. The inhibition of AChE results in the accumulation of acetylcholine in the synaptic cleft, causing hyperexcitation at the central nervous system and disturbance of normal physiological functioning. Another mechanism of toxicity attributed to the CPF is the generation of reactive oxygen species (ROS) leading to an oxidative stress condition (Yu et al., 2008; Goel et al., 2005; Rodrigues et al., 2019; Gomes et al., 2020). Chlorpyrifos was shown to alter the motor activity accompany by DA neurons damage in the midbrain substantia nigra. Moreover, CPF affected the tyrosine hydroxylase (TH) expression. Hence, suggesting that CPF can pose a risk for the development of Parkinson's disease (PD) (Zhang et al., 2011; Sheikh & Sheikh, 2020). Chlorpyrifos was shown to affect the expression of

TH, dopamine transporter, and the genes that metabolize dopamine in rats (Eells & Brown, 2009; Ibrahim et al., 2020), and cell lines (Xu et al., 2012).

1.3.4 Pyrethroids

Pyrethroids are insecticides that were synthesized based on natural chrysanthemum pyrethrins which are isolated from the Chrysanthemum genus of plants (Kolaczinski & Curtis, 2004). They appear to be a better option than organophosphates and carbamates, as they are less persistent and poisonous. They are currently widely employed in agriculture, and they are the most commonly supplied for household use, either to control indoor pests or to prevent head lice in humans and mites in domestic animals. Pyrethroid pesticides are generally made from three potential compounds: deltamethrin (DLM), permethrin, and cypermethrin, commonly supplied and advised for use at home because they are relatively non-toxic to humans (Chrustek et al., 2018). However, recent studies have shown that they are not completely safe for human health, as they are considered neurotoxic substances. Pyrethroids affect the neurological system by interacting with sodium channels causing more sodium ions to pass resulting in prolonged depolarization in neuronal cells and altering the release of neurotransmitters (Figure 3), however, the effect of pyrethroids on neurotransmitter release may be dual stimulatory or inhibitory, or both (Hossain et al., 2004).

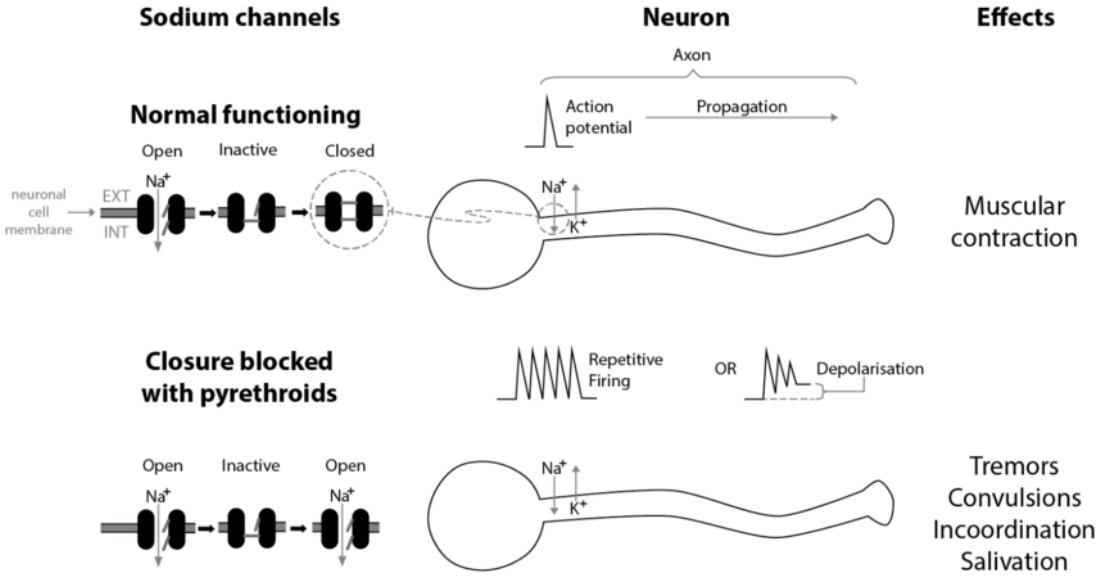

Figure 3: Pyrethroids' mode of action on neurons (Hénault-Ethier, 2015).

Two types of pyrethroids can be identified based on acute intoxication symptoms: (a) Type I, which alters sodium channel opening and closing in neuronal membranes. It causes tremors all over the body, as well as aggressive behavior, and motor ataxia; and (b) Type II, which affects the sodium, chloride, and GABA channels, causing salivation and motor dysfunction (Lucero & Muñoz-Quezada, 2021).

The main concern associated with pyrethroid exposure is the development of progressive neurodegenerative disease (NDs). Deltamethrin, for example, can reach the brain in amounts that are probably toxic due to its lipophilic properties (Mohammadi et al., 2019). Early exposure to pyrethroids in rodents has been shown to cause long-term changes in cholinergic and behavioral variables, in addition to the possibility that they target the dopaminergic and serotoninergic systems. Pyrethroids exposure has been found as a major risk factor for NDs in several epidemiological investigations (Rodriguez et al., 2016).

1.3.5 Deltamethrin

Deltamethrin (Figure 4) is a synthetic Type II pyrethroid with broad-spectrum activities against insects. It is widely used to protect crops, golf courses, additionally it is used against animals' ticks and to control vector-borne diseases. Deltamethrin has become one of the most widely used insecticides in the world due to its high efficiency and low persistence in soil. However, human and animal exposure occurs by either direct contact with vapors or the ingestion of contaminated food, or water and leads to serious health problems (El Golli-Bennour et al., 2019; Han et al., 2020).

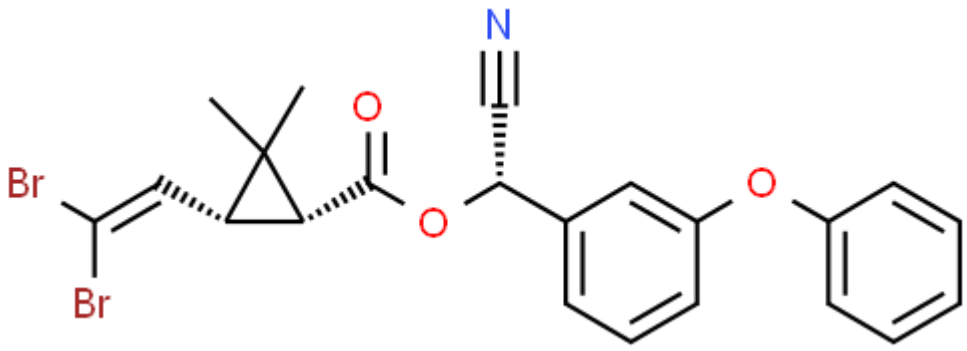

Figure 4: The chemical structure of DLM (chemspider.com)

The effect of long-term exposure of even low doses of DLM was mostly examined in epidemiological and animal studies (Tewari et al., 2018). One study indicated that DLM exposure could induce thyroid dysfunction and behavioral disorders in adolescent mice (Zhang et al., 2020). Another study on quail found that *in vivo* treatment with DLM-induced liver fibrosis in a dose-dependent manner through the promotion of oxidative stress. (Han et al., 2020). The main mechanism of DLM as an insecticide like other pyrethroids is due to its ability to bind to voltage-gated sodium channels receptors and thus, prolonging the open state by inhibiting channel

deactivation and inactivation resulting in tremor followed by the death of insects. However, due to its high hydrophobicity, it could exert other effects on biological membranes at sites other than the voltage-dependent sodium channel (Abdel-Daim et al., 2013) like chloride, and calcium channels (Romero et al., 2015). Many studies on the side effects of this insecticide have been reported, including allergy and immunosuppression, cardiovascular, and reproductive side effects. Besides, hepatotoxicity and nephrotoxicity have been also induced. Several studies have reported that oxidative stress has a role in DLM-induced toxicity in rat's brains (Li et al., 2011; Mohammadi et al., 2019). Antioxidants are proven to be effective in ameliorating DLM-induced toxicity in many previous interventions (Abdel-Daim et al., 2013). Deltamethrin also interferes with the mechanisms of dopaminergic neurotransmissions. The alterations of the dopaminergic pathway caused by exposure to DLM are suggested as a risk factor for PD (Souza et al., 2018).

1.4 Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disorder affecting more than 1% of the population over the age of 60. The selective and progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) is the main characteristic of PD (De Lau & Breteler, 2006; Fereshtehnejad & Lökk, 2014). An additional pathological distinguishing mark of this disorder is the presence of cytoplasmic inclusions within the surviving dopaminergic neurons which are called Lewy bodies, and they have mainly consisted of α-Synuclein and ubiquitin among other proteins (Lotharius et al., 2002). Abnormal protein aggregation, oxidative damage, and mitochondrial dysfunction are involved in the formation and progression of PD. Additionally, decreased glutathione peroxidase activities, catalase, and a

reduction of glutathione (GSH) have been also observed in PD patients (Figure 5) (Farooquinn & Farooqui, 2011; Nagoshi, 2018; Wang et al., 2020). Motor symptoms such as bradykinesia, rigidity, postural instability, and resting tremor as well as non-motor symptoms including sleep disturbances, mood disorders, and cognitive impairments, are associated with Parkinson's patients (Engineering et al., 2014).

Parkinson's disease cases that occur in individuals without having positive family history are defined as "sporadic PD". However, this categorization does not exclude genetic factors as causative agents. About 95% of the PD cases are sporadic and it is believed that sporadic PD is multifactorial with both genetic and environmental contributions (Subramaniam et al., 2013). Currently, there are no established curative or preventative interventions because the understanding of the molecular mechanisms of pathogenesis is not completely known (Nagoshi, 2018). Although dopaminergic neurons account for less than 1% of neurons in the brain (Karam et al., 2020), their degeneration and loss will lead to the development of PD.

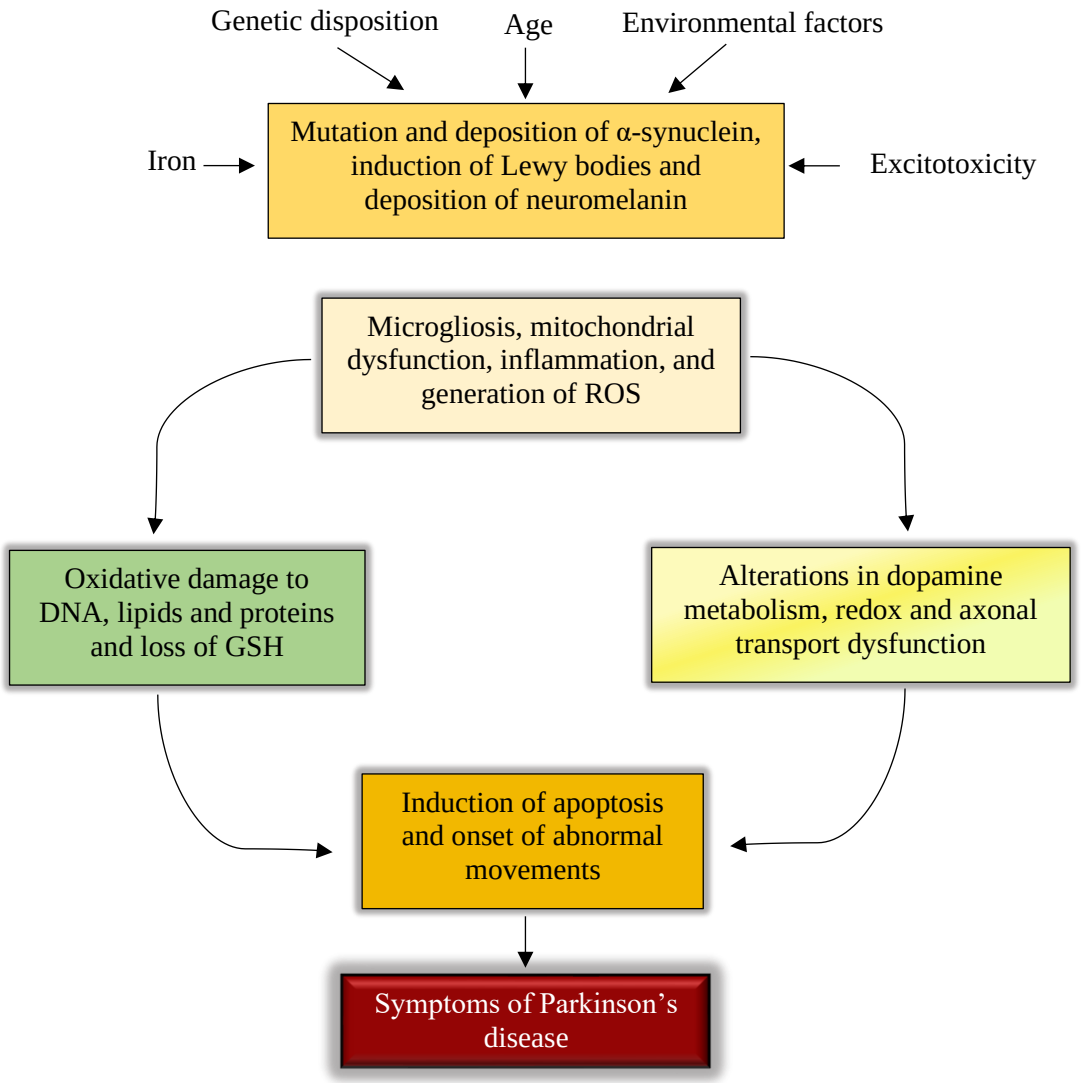

Figure 5: Potential factors and events associated with the pathogenesis of PD. This figure was reproduced from (Farooqui & Farooqui, 2011).

1.4.1 Dopamine and Parkinson's Disease

Dopamine (DA) is a neurotransmitter that coordinates locomotor activity in both vertebrates and invertebrates (Riemensperger et al., 2013). it is made in the brain by an enzyme called TH that converts the amino acid tyrosine to dihydroxyphenylalanine (dopa), which is converted to DA by the enzyme aromatic L–amino acid decarboxylase sometimes termed dopa-decarboxylase (*DDC*) (Figure 6) (Thoener et al., 2021). It is estimated that at least 60% of the SNpc dopaminergic

neurons should be lost and a significant depletion (up to 85%) of DA levels should be observed in the nigrostriatal system before PD becomes clinically evident (Dauer & Przedborski, 2003). Dopamine is a naturally occurring catecholamine whose actions are mediated by G protein-coupled DA receptors. In humans, D1- and D5- receptors constitute the D1-subfamily and exert an excitatory effect by activating adenylyl cyclase, whereas members of the D2-subfamily, i.e., the D2-, D3-, and D4-receptors, inhibit neuronal activity by inhibiting adenylyl cyclase or by coupling to different intracellular second messenger systems (Civelli et al., 1993). D1, D2, and D3 receptors are involved in reward and reinforcement mechanisms. Both D1 and D2 receptors seem to be critical for learning and memory mechanisms (Xu et al., 2009). At the same time, D3, D4, and potentially, D5 receptors seem to have a minor modulatory influence on some specific aspects of cognitive functions that are mediated by hippocampal areas. The roles of D4 and D5 receptors, which have a limited expression pattern in the primary motor regions of the brain, seem to have a minimal role in the control of movement (Sokoloff et al., 2006; Beaulieu et al., 2011). Pharmacological agents targeting dopaminergic neurotransmission have been clinically used mainly as antiparkinsonian drugs and antipsychotics (Roth et al., 2004). Although the main function of DA is in the central nervous system, DA receptors are also found to have other important functions in peripheral locations, including the pituitary and parathyroid glands as well as the kidney (Aperia, 2000; Witkovsky, 2004; Li et al., 2006). Dopamine has a critical role in movement coordination. Therefore, DA deficiency in the brain, movements may become delayed and uncoordinated.

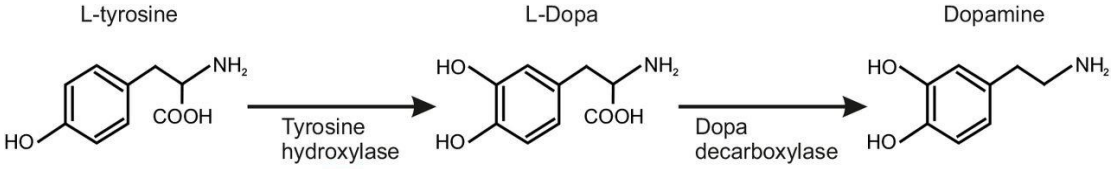

Figure 6: Synthesis of DA (Thoener et al., 2021).

1.4.2 Acetylcholine and Parkinson's Disease

Acetylcholine is another neurotransmitter that is needed for proper locomotion and cognitive performance. Consequently, deficits in ACh synaptic transmission led to defects in motor and cognitive regulation. Therefore, ACh balance is essential for the normal function of the nervous system (Showell et al., 2020). The classical role of AChE is to terminate the transmission of the Ach through the rapid hydrolysis, resulting in the buildup of the neurotransmitter on the synapses, therefore, a net increase in cholinergic signal transmission in cholinergic receptors and postsynaptic cells. Parallel to the behavioral parameters, the activity of AChE is well described as being used in the verification of the efficacy of treating PD and other NDs (Araujo et al., 2015).

Dopamine has dual functions, it can exert either excitatory or inhibitory effects on neurons, depending on the receptor that it binds. At the same time, ACh from cholinergic neurons in the striatum counteracts this DA effect. The disruption of this balance between the striatal DA and ACh systems is thought to play a key role in the pathogenesis of neurotoxin-induced PD models. However, recent studies have reconsidered the role of ACh in striatal modulation. For example, it is generally believed that the activation of different subtypes of DA receptors will have an opposite effect on the release of ACh in the striatum (Zhang et al., 2013).

1.5 *Drosophila Melanogaster*

The arthropod *Drosophila melanogaster* (fruit fly) is a versatile model organism that has been used to study disciplines ranging from fundamental genetics to the development of tissues and organs (Ugur et al., 2016). Throughout the last century, it was used extensively as an animal model for biological research in the disciplines of genetics, molecular biology, and cell biology. (Jana et al., 2016). Although humans and *Drosophila* may not look very similar, most of the pathways that control survival and development are conserved through evolution and it was shown that approximately 75% of known human disease genes are present in *D. melanogaster* consolidating its legitimacy as a model organism for medical research (Pandey et al., 2011). Compared with other models, many technical advantages of using *Drosophila* over vertebrate models; they are easy and inexpensive to culture in laboratory conditions due to their tiny body size and short lifespan, they offer rapid generation time because of their short life cycle, their genome can be manipulated easily and in numerous ways, as they do not have ethical concerns like using other vertebrate models (Baenas et al., 2019). *Drosophila* has been an excellent model organism to study the molecular mechanisms of diverse human diseases including cancer, inflammation, and metabolic disorders, cardiovascular disease, diabetes, asthma-related diseases, and NDs *in vivo*, by using sophisticated genetic techniques (Pandey et al., 2011; Roeder et al., 2012; Yamamoto & Seto, 2014; Engineering et al., 2014; Cheng et al., 2018; Mirzoyan et al., 2019;).

1.5.1 *Drosophil*a as Model Organism for Testing Neurotoxicity

In recent years *Drosophila* has been used as a potential model organism to study either toxin-induced or genetically linked PD. Furthermore, *Drosophila*

melanogaster has been considered as a valuable model for the identification of pharmacological properties of plants and plant-derived constituents against chemical-induced oxidative stress (Sudati et al., 2013; Panchal & Tiwari, 2017; Farombi et al., 2018).

In *D. melanogaster*, DA signaling modulates several vital behaviors similar to mammalian systems including locomotion, learning and memory, reward, and drug response (Nichols, 2006; Cassar et al., 2015; Karam et al., 2020). A reduction in dopaminergic neurotransmission is known to interfere with the behavior and lead to a reduction in locomotor activity (Hanna et al., 2015, Sudati et al., 2013). Although fruit flies seem to be completely unrelated to humans, it was found that several genes involved in DA dynamics (synthesis, secretion, transport, and metabolism) and signal transduction (receptors and downstream signaling cascades) are conserved between fruit flies and humans. Similar to humans, fruit flies can synthesize DA from tyrosine via two enzymatic steps. The first rate-limiting step is the conversion of tyrosine into l-3,4-dihydroxyphenylalanine (l-DOPA) by TH, encoded by the pale (*ple*) gene in *D. melanogaster*, followed by the second step where l-DOPA is converted to DA by decarboxylase enzyme, encoded by the DOPA decarboxylase gene (*ddc*) (Figure 7) (Cichewicz et al., 2017; Karam et al., 2020). Dopamine transporter (*dat*), an integral component of normal functional DA that mediates the termination of DA neurotransmission by rapid reuptake of DA into the presynaptic terminal (Figure 7). Several studies have demonstrated the role of *dat* in affecting the vulnerability of the DA neuron to neurotoxins. Therefore, upregulation of *dat* activity may increase the susceptibility of DA neurons to exogenous neurotoxicants by increasing their uptake by *dat* (Elwan et al., 2006; Yamamoto & Seto, 2014). Fruit flies express four forms of G-protein coupled DA receptors, two (D1-like receptors, *dop1r* and *dop1r2*) are

orthologs of mammalian D1 receptors, the third (D2-like receptor *dop2r*) is a functional ortholog of the mammalian D2 receptors and the fourth one is a non-canonical receptor (*dopecr*) which is homologous to mammalian β-adrenergic receptors, it binds ecdysone, an insect steroid hormone. This binding in turn can negatively regulate the DA-mediated activation of *dopecr in vitro*. (Yamamoto & Seto, 2014; Karam et al., 2020). D1-like receptors act through activation of the cyclic adenosine monophosphate (cAMP) pathway, while D2-like receptors inhibit this pathway similarly to humans where several pharmacological agents (agonists and antagonists) that target mammalian D1 and D2 receptors have been identified to activate and inhibit *Drosophila* homologs *in vivo* (Yamamoto & Seto, 2014). Furthermore, many drugs that target the mammalian dopaminergic system were also found to be effective in *Drosophila*. A study on transgenic fruit flies to test the locomotor response to prototypes of the main categories of medicine presently used to treat PD was done and they found that administering L-dopa which is a standard treatment for PD in humans was shown to be effective in rescuing *Drosophila* climbing abilities, those were geotactic deficit because of putatively expressed α-synuclein. Similarly, the DA agonists bromocriptine, and pergolide were considerably effective. Dopamine breakdown in mammals and fruit flies differs greatly, in comparison to the highly conserved processes of DA synthesis, secretion, and signaling. In *Drosophila*, no direct orthologs of monoamine oxidase (MAO) and Catechol-O-Methyltransferase (COMT) genes (mammalian metabolic enzymes responsible for inactivation of DA). Instead, arylalkylamine N-acetyltransferase1 (*aanat1*), is thought to be the enzyme responsible for DA metabolism mainly through acetylation (Yamamoto & Seto, 2014). Because fruit flies are amenable for large-scale genetic and chemical screening, they are providing opportunities for understanding the genetic and molecular basis of

diseases, and therefore they could be a useful tool to discover new genes that could be involved in PD and therefore discovering a new therapeutic compound that could be relevant to alleviate PD symptoms in humans (Muñoz-Soriano, 2011; Fernández-Hernández et al., 2016). Moreover, fruit flies are capable of performing complex motor behaviors such as climbing and flying, and their brains are complex enough to make these behaviors relevant to humans. Although it's impossible to completely recapitulate the key neuropathological features of human PD using a single model organism, many of the PD models that are presented in *Drosophila* share key features of the disease and have provided insights into PD pathogenesis. Furthermore, the relatively short life cycle and lifespan of flies (approximately 10 days and three months, respectively) accelerate the study of age-related disorders, including PD (Nagoshi, 2018). Subsequently, fruit flies can undercover the molecular function of human disease genes *in vivo*, expanding our comprehension of the pathophysiology of DA-related neuropsychiatric disorders. Moreover, by identifying new conserved genes that direct DA dynamics and signaling, *Drosophila* research is likewise ready to offer novel candidate genes for neurologic diseases without a known genetic cause as well as additional targets for drug therapy (Pendleton et al., 2002; Nichols, 2006). Pharmacological insults can cause *Drosophila* to have Parkinsonian-like phenotypes, thereby modeling sporadic PD. (Nagoshi, 2018). All of these can solidify the fact that *Drosophila* can be used as a model to represent Parkinson’s phenotype in human ortholog.

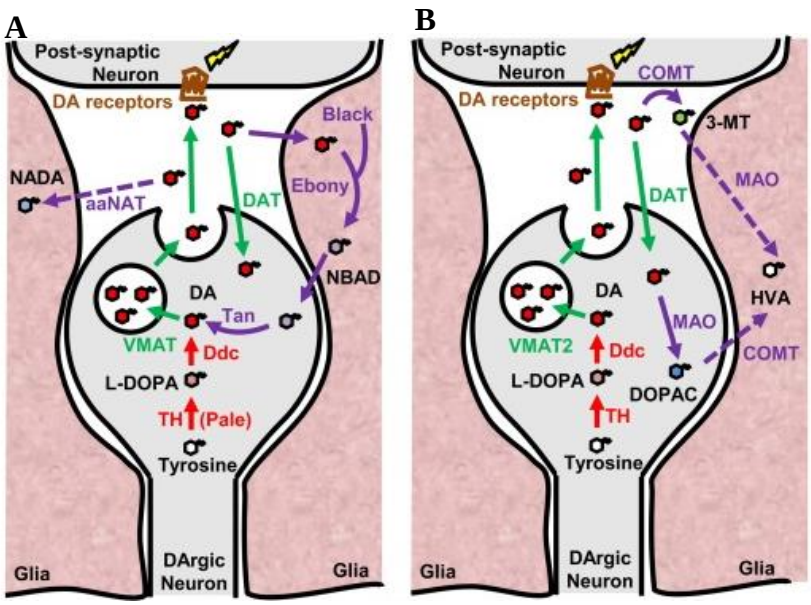

Figure 7: Schematic diagrams of DA dynamics and signaling in (A) *Drosophila* brain, and (B) mammalian brain (Yamamoto & Seto, 2014).

1.5.2 Negative Geotaxis in *Drosophila melanogaster*

Under normal conditions, *Drosophila* exhibit a tendency to climb against gravity, this natural behavior is called negative geotaxis. In *D. melanogaster*, negative geotaxis has been studied for more than a century as an intrinsic part of its locomotor behavior. Negative geotaxis is the measure of how rapidly *Drosophila*, as part of their innate escape response, can climb upwards towards the top after being tapped to the bottom of a vessel or a vial. Researchers have been using this escape reflex widely, taking the advantage of this inherent trait of *D. melanogaster* when startled (Linderman et al., 2012; Taylor & Tuxworth, 2019). The climbing response is generally rapid, and any failure to get over the "shock" after being knocked down reveals defects in the nervous system to control initiation. Several studies have considered measuring negative geotaxis as one of the parameters to study the effects

of different natural compounds and extracts as neuroprotective agents to modulate the toxic effect of neurotoxic chemicals (Sudati et al., 2013; Gomes et al., 2020).

The most common negative geotaxis method is a relative measurement by calculating the number of flies passing a line placed at a certain height (8 cm for example) in the test vial (Feany & Bender, 2000; Ali et al., 2011). Multiple protocols have been developed for analyzing *Drosophila* adult climbing behavior to improve this assay, e.g., Rapid Iterative Negative Geotaxis (RING) assay, which allows high-throughput analysis over numerous flies at the same time (Gargano et al., 2005; Ali et al., 2011).

1.6 Potential Neuroprotective Effects of Natural Compounds

Neuroprotection is the preservation of the function and structure of neurons from cellular injuries induced by a variety of agents or NDs. The onset of the symptoms of NDs including Alzheimer's, Parkinson's, and others are usually gradual as well as progressive, and these NDs affect millions of people around the world with the main risk factor is advancing age. Hence, the oxidative stress generated in the brain caused by the free radical can lead to protein, DNA, and RNA oxidation, and neuronal dysfunction or death. Bioactive compounds that possess properties able to counteract oxidative stress received particular attention in the past years as they can serve as suitable prophylactic and/or therapeutic candidates on the development and/or progression of chemical-induced PD. Moreover, they can serve as food supplements to prevent diseases (Farombi et al ., 2018; Siima et al., 2020) including NDs (Srinivasan et al., 2007). Several clinical trials have shown promising results of the natural products as neuroprotective agents targeting the treatment of NDs (Potashkin & Seidl, 2011; de Andrade Teles et al., 2018). Traditional medicinal plant use is

essentially a spiritual and traditional method of curing a disease (Petrovska, 2012; Chougouo et al., 2016). Alkaloids, glycosides, coumarins, flavonoids, steroids, anthocyanins, fatty acids, tannins, emodins, and leucoanthocyanins, among others, are abundant metabolites in plants/plant-derived components and are responsible for the therapeutic properties of the various plants (Savithramma et al., 2011). Several metabolites of medicinal plants, particularly "secondary metabolites," play a unique role in the treatment of various diseases such as chronic and progressive NDs, diabetes, and cancer (Modak et al., 2007; Singhal et al., 2012; Sengupta et al.,2016). Plant-derived medicines improve human health without causing significant side effects, and about 80% of the world's population in developing countries still rely on plants and plant-derived medicines (Mahmoud & Gairola, 2013; Hosseinzadeh et al., 2015).

1.6.1 Ferulic Acid

Ferulic acid (FA), a naturally occurring phenolic compound that belongs to the hydroxycinnamic acid family, is found mainly in plants. It is a well-studied natural molecule with powerful neuroprotective effects. Ferulic acid is abundant in the leaves and seeds of many plants, especially grains such as brown rice, whole grains, and oats (Pandey & Rizvi, 2009) (Figure 8). Many pharmacological properties are attributed to it, including anti-inflammatory, antioxidant, and neuroprotective activities. Many experimental studies have reported the neuroprotective effects of FA in brain injury, spinal cord ischemia, and Alzheimer's disease (AD) (Abdulwanis Mohamed et al., 2019). Ferulic acid is well known for its strong membrane antioxidant due to its free radicals scavenging effect so, FA can play an important role in therapeutic usage against various diseases. Recent evidence suggests a wide range of therapeutic effects against various diseases like type 2 diabetes, obesity, cardiovascular as well as

neurodegenerative diseases. (Srinivasan et al., 2007; Alam, 2019). Importantly, a recent study has shown that FA attenuated neuroinflammation and improved behavioral deficits against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (Li et al., 2020). Another study has shown a promising neuroprotective effect of FA against rotenone-induced neurodegeneration in the rat as a model for PD through its antioxidant and anti-inflammatory properties (Ojha et al., 2015).

In the literature, there is also evidence of the neuroprotective effect of γ-oryzanol, related to one of its constituents, the ferulic acid. This polyphenol has a protective function against NDs such as AD, and PD (Araujo et al., 2015). In addition to its antioxidant and anti-inflammatory effects, FA also regulates various neuro-signaling pathways via interaction with multiple receptors or enzymes. It also regulates the expression of various pro-inflammatory cytokines and pro-apoptotic signals, which explains its neurotherapeutic effect. Ferulic acid also showed antiparkinsonian effects in a rat model of PD induced by rotenone through the regulation of the levels of heat shock protein (HSP). It provides neuroprotection by significantly increasing TH in the brain (Thapliyal et al., 2021).

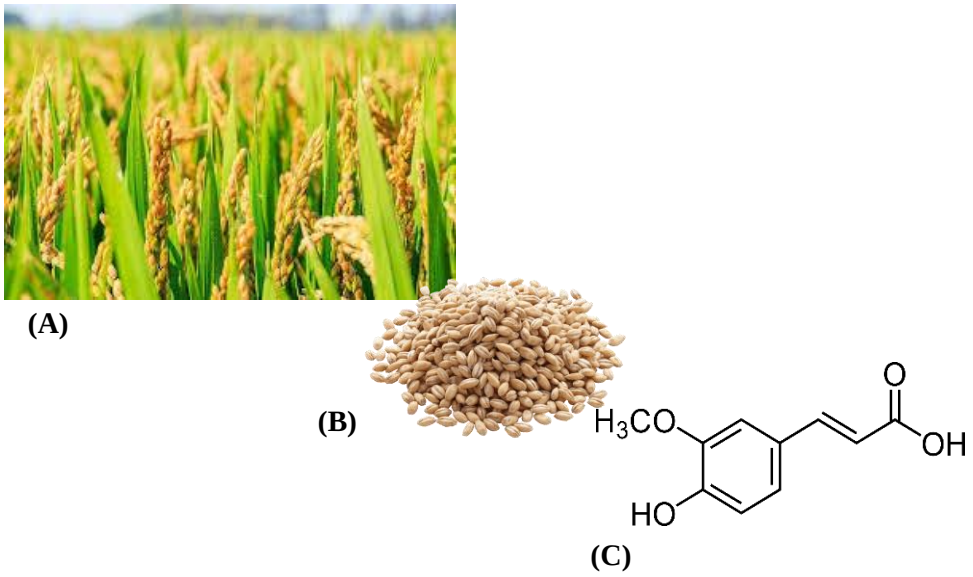

Figure 8: Grain (wheat) (A), its seeds (B); and the chemical structure of bioactive component of seeds, FA (C).

1.6.2 Thymoquinone

Nigella sativa (NS) (black cumin) is the seed of a flowering plant in the Ranunculaceae family (Figure 9), commonly found in the Middle East, and Africa. Its derivatives are widely used as spices and preservatives in the food industry. It is one of the most valuable nutrient-rich medicinal plants with great therapeutic properties such as anti-inflammatory, anti-diabetic, and anti-tumor activities (Ramadan, 2007).

Thymoquinone (TQ) is one of the major bioactive components of NS essential oil. It has a variety of pharmacological effects, including antioxidant, anti-inflammatory, antibacterial, and anti-tumor effects. It has also neuroprotective activities. According to reports, the neuroprotective effect of TQ is due to its ability to reduce the levels of intracellular reactive oxygen species (ROS) in neuronal cells, and to downregulate proinflammatory cytokines (Khan & Afzal, 2016; Isaev et al., 2020). The potential of TQ to protect dopaminergic neurons in cell culture from MPP+ and rotenone cytotoxicity has been previously reported (Sedaghat et al., 2014). Recently,

the ameliorative and inhibitory effects of TQ on PD have also been demonstrated, paving the way for more extensive research on the neuroprotective effects of nutraceuticals (Samarghndian et al., 2018). Various *in vitro* cell line studies and *in vivo* rat studies have reported pathological improvement of AD after TQ treatment. Thymoquinone has a beneficial effect through mechanisms such as scavenging ROS, inhibiting AChE activity, and preventing neurotoxicity (Cascella et al., 2018).

The therapeutic effect of TQ in animal models of PD exposed to rotenone was studied. Co-administration of rotenone and TQ has been shown to suppress Parkinson's symptoms, including dyskinesia caused by rotenone (Farkhondeh et al., 2018). Thymoquinone has been shown to protect midbrain dopaminergic neurons from cell death induced by 1- Methyl-4-phenylpyridinium (MPP+). Another study showed the therapeutic effect of TQ on cell death in primary dopaminergic cultures induced by MPP + and rotenone (Radad et al., 2009).

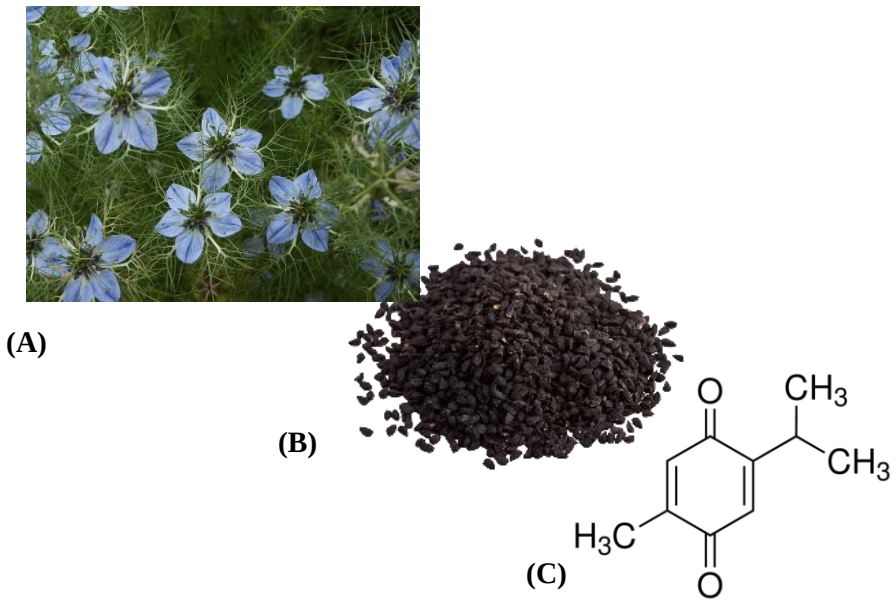

Figure 9: The Nigella sativa plant (A), its seeds (B); and the chemical structure of bioactive component of seeds, TQ (C).

1.6.3 Combination Therapy

Combination therapy or multi-component therapy, in which two or more therapeutic agents are used together, commonly has one or more of the following goals: (i) Reducing the dose, while having non-overlapping toxicity and comparable treatment profile, in order to achieve a curative effect with fewer side effects (ii) Improve effectiveness by exploiting additivity or better yet, greater-than-additive effects in the biochemical activities of the two agents. Combination therapy aims to achieve biological interaction and reduced toxicity (Fitzgerald et al., 2006). According to the McGraw-Hill Concise Dictionary of Modern Medicine, synergy is "the cooperative interaction between two or more components of a system, such that the combined effect is greater than the sum of each part" (Segen, 2006).

Since various antioxidants can result in unpredictable activity, the activity of their mixtures is still an interest for more investigations. It is important to study the effects of interactions between natural products to reduce the amount required to obtain the same result (Capitani et al., 2009).

Understanding how mixtures work together to produce specific biological effects may respond to the growing threat of disease resistance. In fact, many diseases are not regulated by a single molecular target but often have multi-factor causality. Many studies have shown that the disease resistance of a combination of compounds is less than that of a single active ingredient. Plants have been developed for thousands of years to target pathogens through the combined action of structurally and functionally different components to combat disease pathogenesis. Complex natural product mixtures provide important resources for drug development and to ensure future success in natural products research. Understanding the interactions within and

between the components of natural product mixtures is critical. The pharmacological investigations of the combined effect can be studied at the level of molecular targets, disease pathways, cellular processes, and patient responses. Therefore, *in vitro*, in *vivo*, preclinical, and clinical studies can provide valuable insights into the combined effects (Caesar & Cech, 2019).

1.7 Gene Expression and RT-qPCR

Understanding the molecular mechanisms is necessary to elucidate common human diseases including NDs, thereby, improving diagnostic tests and disease management. Assessing differential gene expression can help to precisely describe these disease-associated pathways, by establishing prognostic signatures of disease progression and drug response. Recent gene expression studies have involved biological pathways such as inflammation, protein homeostasis, RNA splicing, as the key features in the development and progression of multiple NDs, such as PD, and Alzheimer's disease (AD) (Schadt et al., 2005; Simunovic et al., 2008). Quantitative real-time polymerase chain reaction (RT-qPCR) is one of the most widely used techniques to study and analyze low abundance gene expression derived from various sources for discovering disease-associated alterations. The RT-qPCR is highly sensitive and provides the necessary accuracy and reliability as well as it is easy to perform and allows quantification of rare transcripts and small changes in gene expression (Pfaffl, 2001). Studies on NDs are no exceptions where RT-qPCR has helped to identify the changes in gene expression of several disorders such as PD (Rydbirk et al., 2016).

Chapter 2: Methods

2.1 *Drosophila* Stock, Diet, and Rearing

Drosophila melanogaster, wild-type (Oregon R strain) were obtained from a colony maintained in the Entomology laboratory at the Biology Department, United Arab Emirates University, which was originally procured from the United States of America (Carolina Biological Supply, Burlington, NC). The flies were maintained and reared on instant *Drosophila* medium Formula 4-24® (Carolina Biological Supply, Burlington, NC) under controlled temperature (23±1°C) and 12:12 h light/dark cycle

2.2. Feeding Device

This device (Figure 10) was assembled from a plastic FisherbrandTM *Drosophila* vial (FisherScientific, USA) Cat. No. AS513 and a cotton swab Sky OrganicsTM, Cat. No. SYO-00765 (Company name), which was inserted into a hole made in a foam plug FisherbrandTM *Drosophila* BuzzPlugsTM (FisherScientific, USA) Cat. No. AS275. The hole was made using a thin metal nail and was small enough to allow the swab to fit snugly.

2.3 Experimental Protocol of Exposure to CPF for 24 Hours

Each experiment included three feeding devices (Figure 10) for each treatment (CPF and control). The CPF stock solution was prepared by dissolving CPF in DMSO. Deionized distilled water was used to prepare the 10% sucrose solution, which was used as a control after adding a DMSO amount equal to one present in the CPF solution. The DMSO concentration in the control (water) did not cause any significant mortality compared to pure water (data not shown). The two treatments were 10%

sucrose solution containing DMSO (vehicle) as control and 10% sucrose solution containing 2 μM CPF. The CPF concentration used in this study was selected based on a serial dilution bioassay study (data not shown). The median-lethal concentration (LC50) values for different CPF concentrations were calculated using the AAT Bioquest® calculator (https://www.aatbio.com/tools/lc50-calculator). Each cotton bud was moistened with 700 μL of 10% sucrose solution of the appropriate treatment. Age synchronized adult (4-6 days old) male fruit flies (n = 40) were segregated under brief cold anesthesia and transferred into each feeding device. In all cases, the experiments were done in triplicate and repeated three times (n = 3).

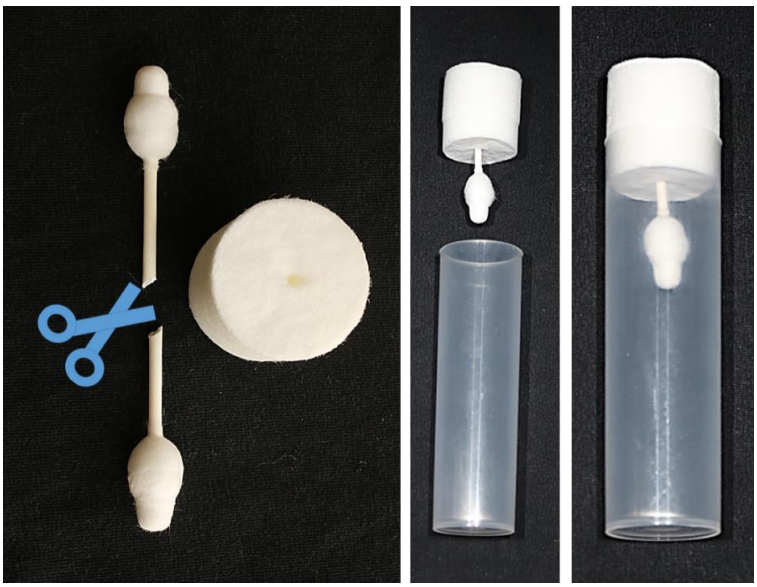

Figure 10: *Drosophila melanogaster* cotton swab feeding device.

Left: one cotton swab was cut into two halves using a pair of scissors and a hole was made in a foam plug; Middle: one half of the cotton swab was inserted in a hole made in a foam plug; Right: the assembled feeding device.

2.4 *In Vivo* Assays

2.4.1 Survival

After introducing the fruit flies into the feeding devices, that contain cotton bud soaked with the appropriate treatment, they were monitored, and mortality was recorded after 24 hours. Flies, incapable of coordinated movement, after gentle touching with a thin paintbrush, were considered dead. Surviving flies were then subjected to negative geotaxis assay and afterward to different molecular and biochemical assays.

2.4.2 Negative Geotaxis Assay

The negative geotaxis was assayed by three different methods: the 8-cm method, RING method, and modified RING method (8 seconds method). Each method was repeated five times at 1 min intervals using the same insects; the score for each replication was an average of the five-time trials. Every assay was repeated three times using three different groups of insects. Fruit flies were collected using a manual aspirator and transferred to a vertical polystyrene vials (length, 9.5 cm; diameter, 1.5 cm) sealed and taped securely by another tube near the contacting openings, and then the tubes were set in a custom-made holder designed to tightly hold 7 tubes upright in a row. 8 cm above the bottom on the lower vial was measured and marked. The fruit flies were allowed to acclimatize for 30 min. Fruit flies were gently tapped to the bottom of the column while the camera is recording.

The first method used a climbing apparatus in which two empty polystyrene vials were vertically joined by tape facing each other (openings of the vials were perfectly aligned). Fruit flies were gently tapped down to the bottom of the vial and

the number of flies that can climb above the 8-cm mark by 8 seconds after the tap was measured in an image that was captured using a digital camera. The number of flies that passed the 8-cm mark was recorded as a percentage of total flies (Feany and Bender, 2000; Chaudhury et al., 2007; Ali et al., 2011).

The second method was the RING assay, which was used as described previously by Gargano et al. (2005). The climbing apparatus was a 50 mL plastic tube. Briefly, this technique measures average height climbed by individual flies during a defined time after induction of negative geotaxis. The fruit flies were allowed to ascend the wall of the tube for 3 secs after initiating the behavior. Originally, the distance climbed by each fly was taken visually against a paper ruler attached from outside to the 50 mL tube in an image that was captured using a digital camera. However, in the current study, we used software to measure the distances more accurately and uniformly. A screenshot was taken, and the image was processed by Past Software (https://www.nhm.uio.no/english/research/infrastructure/past/) to calculate the distance climbed by each fly.

The third method used a climbing apparatus, which was also two empty polystyrene vials that were vertically joined by tape facing each other. Fruit flies were gently tapped down to the bottom of the vial and were allowed to ascend the walls of the vials for 8 secs. The distance climbed by each fly was measured in an image that was taken by a digital camera. This method is similar to the RING assay but in a longer climbing apparatus and more climbing time.

2.5 *In Vitro* Assays

2.5.1 RT-qPCR

RNA was extracted from the control and treated flies using Qiagens RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA was measured by Quantus™ Fluorometer (Promega, Madison, USA). Real-Time Polymerase Chain Reaction was performed in triplicates and each reaction contained 50 ng total RNA using Luna® Universal One-Step RT-qPCR Kit (New England Biolabs Inc., Ipswich, USA) in the QuantStudio 5 Real-Time PCR system (Applied Biosystems). All kits were used as per the manufacturer's instructions. Relative transcript level was determined by the 2(-ΔΔCt) method (Livak & Schmittgen, 2001). The following genes from the dopaminergic neurotransmission system were analyzed after CPF exposure: tyrosine hydroxylase (*ple*), DOPA decarboxylase (*ddc*), DA transporter (*dat*), D1-like receptor 1 (*dop1r1*), D2-like receptor (*dop2r*), DA/ecdysteroid receptor (*dopecr*), and DA N-acetyltransferase (*aanat1*) (Table 1). The glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene was used to normalize gene expression (Table 1). Primer sequences were obtained from previous studies or designed with the PrimerBlast tool, based on specific sequences from each gene published in Genebank (http://www.ncbi.nlm.nih.gov).

Table 1: Sequences of RT-qPCR Primers.

Primer	Sequence	Reference
ple	For 5'-AACACCGGATTCTCTCTCCG-3' Rev 5'-CTCGTGAATGGAGTCGGGCT-3'	Present study
ddc	For 5'-ACACAAATGGATGCTGGTGA-3' Rev 5'-AGAGGGTCCACATTGAACG-3'	Norry et al. (2009)
dat	For 5'-GGTGCCCCTCTTCAAAGGAAT-3' Rev 5'-ATTACACGACGTCCAAGGCA-3'	Figueira et al., 2017
dop1r1	For 5'-ACGATGGCACAACGTTGACA-3' Rev 5'-GCACCGATAGGAAGATGCCA-3'	Figueira et al., 2017
dop2r	For 5'-CACAAGGCCTCGAAAAAGAA-3' Rev 5'-GCGAAACTCGGGATTGAATA-3'	Inagaki et al. (2012)
dopecr	For 5'-AGGGTCCTGTGTGTACTGGT-3' Rev 5'-GCAAGAATTGTTGGCTTTTCCG-3'	Figueira et al., 2017
aanat1	For 5'-AACGAATCGGGCGAAAGTCT-3' Rev 5'-CGTTCAGGCGTGAAATTGGC-3'	Figueira et al., 2017
gapdh	For 5'-GCTCCTCAATGGTTTTTCCA-3' Rev 5'-ATGGAGATGATTCGCTTCGT-3'	Figueira et al., 2017

2.5.2 Determination of AChE Activity

Acetylcholinesterase activity was evaluated using the method of Ellman et al. (1961). Samples from treatment and control groups were homogenized as 1:100 (flies(mg)/ volume µl PBS (pH 7.4)+ protease inhibitor cocktail) and then centrifuged at 5000 rpm for 5 min at 4°C. the supernatant was then used to determine AChE activity. The reaction mixture contained 80 µL of PBS (pH 7.4), 50 µL of 0.32 mM DTNB, 20 µL of the sample, and 50 µL of 10 mM acetylthiocholine. Then, the reaction was monitored at a wavelength of 405 nm (for 10 min) in a Platos-R-496-AMP

AMEDA microplate reader (Labordiagnostik GmbH, Graz, Austria). The AChE activity was expressed as a percentage of AChE activity.

2.6 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9 for Windows (GraphPad Software, San Diego, CA, United States). A two-tailed Student's t-test was used to identify differences between the control group and the treatment groups. AChE % activity was analyzed by one-way ANOVA followed by Bonferroni post hoc test. Gene expression data were analyzed using two-way ANOVA followed by Bonferroni post hoc test. Differences with $P < 0.05$ were considered statistically significant. The data were presented as mean ± standard error of the mean (SEM).

2.7 Experimental Protocol of Exposure to DLM

The same protocol that was mentioned for CPF exposure was used for DLM except for the concentration, which was 0.59 µM for DLM.

2.8 *In Vivo* Assays

2.8.1 Survival

Survival was recorded as mentioned previously in CPF.

2.8.2 Negative Geotaxis Assay

One methodology was chosen (the third method; the modified RING assay) as all methods were able to detect the differences in the climbing performance of the fruit flies as will be clarified in the results section.

2.9 *In Vitro* Assays

All molecular and biochemical assays were applied similarly as mentioned previously in CPF.

2.10 Statistical Analysis

The same statistical analysis tools that were used to analyze the data from the CPF experiment were repeated for DLM.

2.11 Experimental Protocol of Exposure to DLM & Neuroprotective Agents

The same protocol that was mentioned for CPF exposure was used for the DLM and neuroprotective agent's experiment. In this experiment, the fruit flies were exposed to different treatments for 72 hours as following: (1) control; (2) 0.59 μM DLM; (3) 250 μM FA; (4) 25 μM TQ; (5) FA+TQ (Mix); (6) DLM+FA; (7) DLM+TQ; and (8) DLM+Mix. All chemicals were dissolved in DMSO. Fresh chemicals were added every 24 hours to avoid desiccation.

2.12 *In Vivo* Assays

2.12.1 Survival

Survival was recorded after 72 hours as mentioned previously in CPF.

2.12.2 Negative Geotaxis Assay

The modified RING assay was used to detect the differences in the climbing performance of the fruit flies untreated, treated with DLM alone, or co-treated with the assigned neuroprotective agents after 72 hours of exposure.

2.13 *In Vitro* Assays

All molecular and biochemical assays were applied similarly as mentioned previously in CPF.

2.14 Statistical Analysis

The same statistical analysis tools that were used to analyze the data from the CPF experiment were repeated for DLM.

Chapter 3: Results

3.1 Chlorpyrifos Exposure

3.1.1 Effect of CPF on the Survival of *D. Melanogaster*

Exposure of male adult flies to 2 µM (0.7 ppm) of CPF for 24 hours resulted in a significant decrease in the percentage of surviving flies 64.2% compared to the control 100 % (P = 0.0037) (Figure 11).

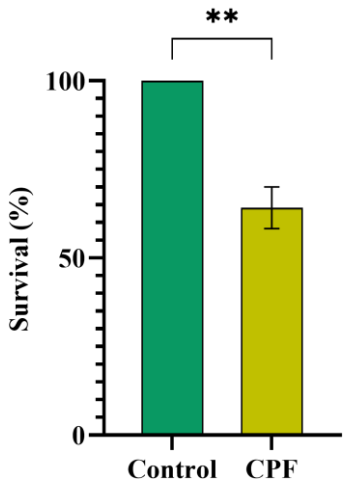

Figure 11: Effect of exposure to CPF on survival of *D. melanogaster*.

The mortality was scored after 24 hours of treatment with 2 µM CPF (0.7 ppm). The total number of flies (120 per group) represents the sum of three independent experiments. The results are represented as mean ± SEM and expressed as the average mortality percentage of flies, Data were analyzed by two-tailed Student's *t*-test. **P ≤ 0.005 significant differences between control and CPF-fed flies.

3.1.2 Locomotor Performance of *D. Melanogaster* Exposed to CPF

We assessed the locomotor performance of adult flies exposed to CPF by quantifying climbing ability by three different negative geotaxis assays (% of flies passing 8-cm mark, RING assay, modified RING assay). All the three methods revealed that the exposure to 2 µM CPF (0.7 ppm) for 24 hours caused severe

locomotor impairment (P= 0.0015, P= 0.0027, P= 0.003, respectively) (Figure 12). Among untreated controls, 86.65% of flies were able to pass the 8 cm within 8 secs compared to 39.06% in CPF treated flies (Figure 11, 2A). The average distance climbed by the same untreated flies was 30.70% and 65.41% of the tubes in 3 and 8 secs, respectively compared to 15.75% and 32.44% in CPF treated flies during 3 and 8 secs, respectively (Figure 12, 2B, 2C). Chlorpyrifos-exposed flies exhibited a significant decrease in climbing ability suggesting the induction of locomotor deficits.

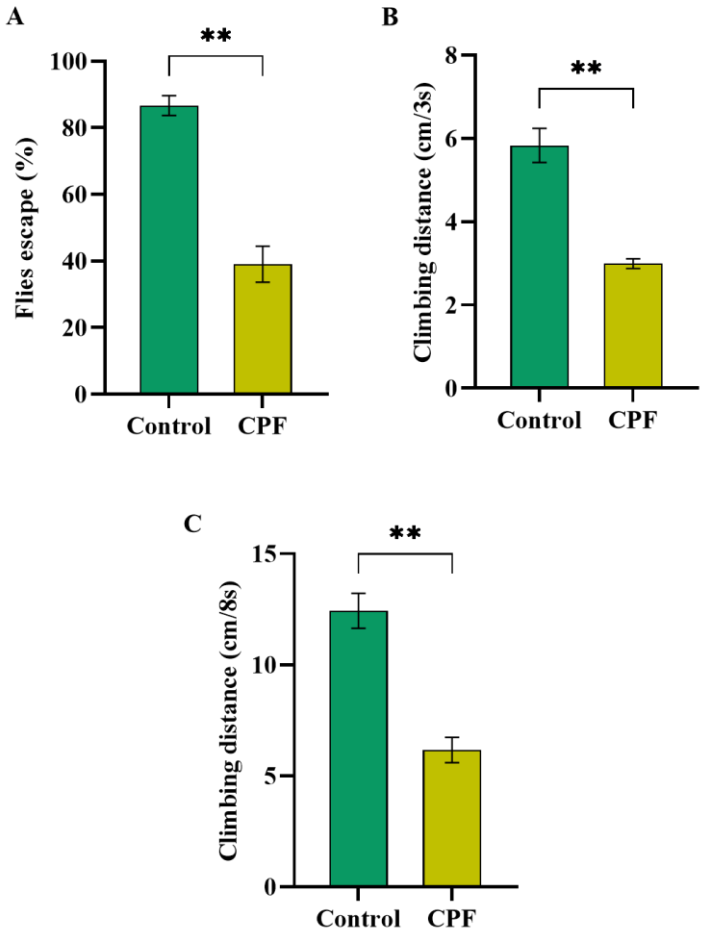

Figure 12: Effect of exposure to CPF on climbing behavior of *D. melanogaster*.

Climbing behavior of *D. melanogaster* adults exposed to 2 µM (0.7 ppm) CPF for 24 hours was determined by: (A) scoring the percentage of flies able to climb 8 cm in 8 s, (B) scoring the average distance (cm) climbed by the flies in 3 s, (C) scoring the average distance (cm) climbed by the flies in 8 s. The total number of survived flies represents the sum of three independent experiments. The results are presented as mean ± SEM. Data were analyzed by a two-tailed Student's *t*-test. **P ≤ 0.005 significant differences between control and CPF-fed flies.

3.1.3 Effect of CPF on The AChE Activity

The AchE, a hallmark for OPs poisoning, was measured. The enzyme activity was significantly inhibited (P = 0.0017) in flies exposed to CPF (25.67% decrease in AchE). As shown in (Figure 13).

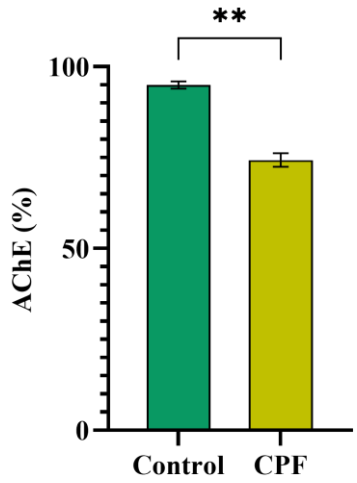

Figure 13: Acetylcholinesterase activity in flies exposed to CPF.

The AchE activity was measured after flies were exposed to CPF 2 µM for 24 h. Data were analyzed by a two-tailed Student's *t*-test. Results are expressed as a percentage of control (mean ± SEM); ∗∗p < 0.005 compared to the CPF group.

3.1.4 Effects of CPF on Gene Expression Profile of Dopaminergic System

We quantified the mRNA of seven genes responsible for DA biosynthesis, transportation, metabolism, and reception in total-RNA extracts using RT-qPCR. Fruit flies exposed to 2 µM (0.7 ppm) CPF for 24 hours showed no statistically significant differences between the control and the treatment groups among all tested genes. However, male flies exposed to CPF decreased in the *ple* mRNA gene expression by 26.1% when compared to control groups. An increase in *ddc*, *dat*, *dop1r1*, *dop2r*, *dopecr*, and *aanat1* expression in males exposed to CPF was 13.0, 38.31, 22.79, 27.77, 25.16, and 60.86%, respectively when compared with the control groups (Figure 14), and (Figure 15).

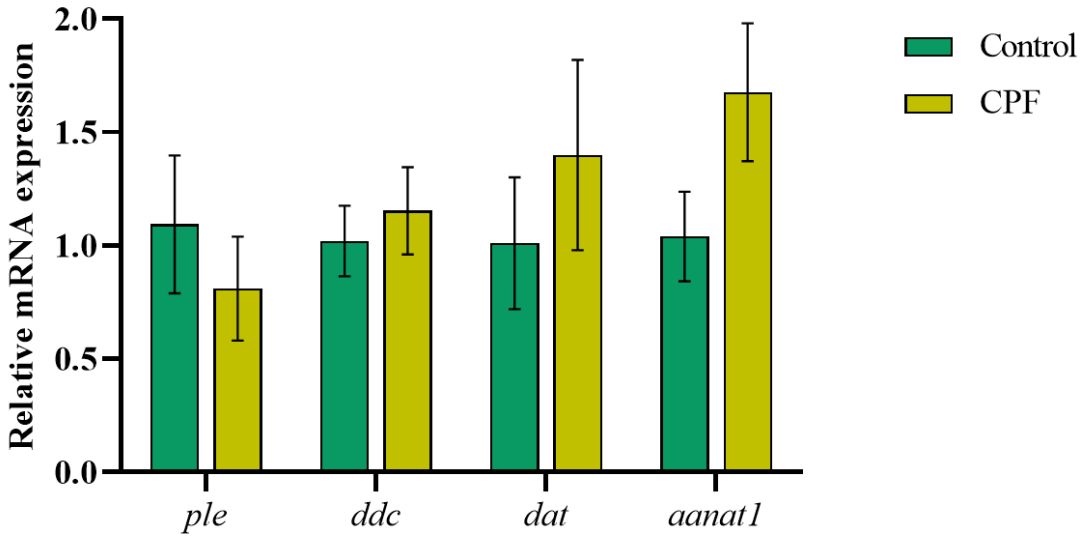

Figure 14: RT-qPCR gene expression of *ple*, *ddc*, *dat*, and *aanat1* in male flies exposed to 2 µM CPF.

Results are expressed as mean fold change ± SEM relative to control flies. Data were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. P ≥ 0.05 indicates no significant differences between control and CPF-fed flies.

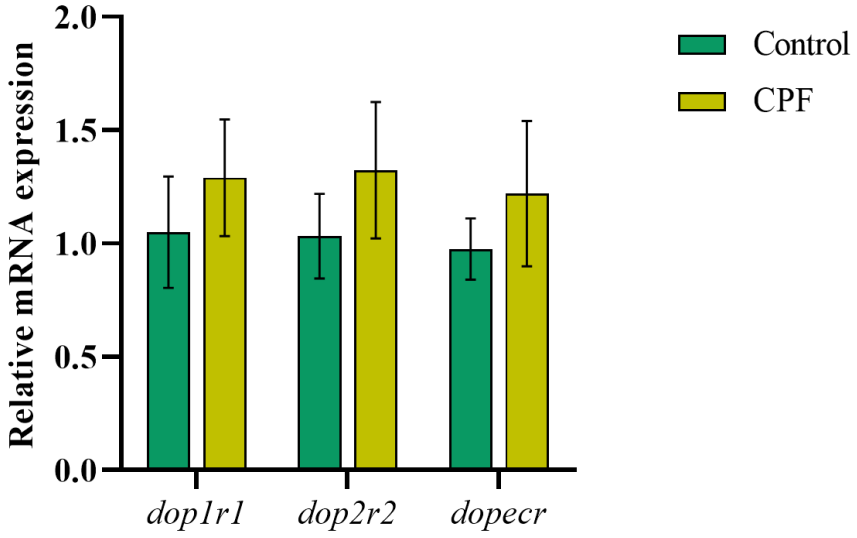

Figure 15: RT-qPCR gene expression of *dop1r1*, *dop2r*, and *dopecr* in male flies exposed to 2 µM CPF.

Results are expressed as mean fold change ± SEM relative to control flies. Data were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. P ≥ 0.05 indicates no significant differences between control and CPF-fed flies.

3.2 Deltamethrin Exposure

3.2.1 Effect of DLM on the Survival of *D. Melanogaster*

Exposure of male adult flies to 0.59 µM of DLM for 24 hours resulted in a significant decrease in the percentage of surviving flies 82.2% compared to the control 100% (P = 0.0047) (Figure 16).

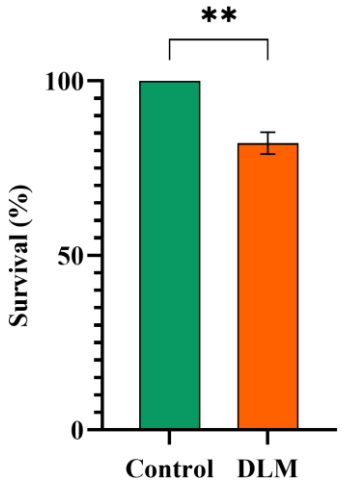

Figure 16: Effect of exposure to DLM on the survival of *D. melanogaster*.

The mortality was scored after 24 hours of treatment with 0.59 µM DLM. The total number of flies (120 per group) represents the sum of three independent experiments. The results are represented as mean ± SEM and expressed as the average survival percentage of the flies, Data were analyzed by a two-tailed Student's *t*-test. **P ≤ 0.005 significant differences between control and DLM-fed flies.

3.2.2 Locomotor Performance of *D. Melanogaster* Exposed to DLM

Wild-type flies exposed to DLM for 24 hours displayed a robust defect in reaching the top of the apparatus as compared to control flies indicating locomotor dysfunction. The average distance climbed by the untreated flies was 11.01 cm compared to 2.70 cm in DLM treated flies during 8 secs (P = 0.0025) (Figure 17).

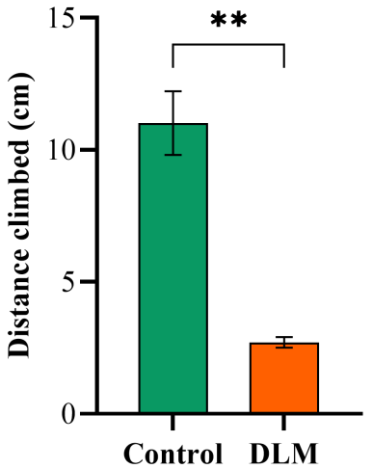

Figure 17: Effect of exposure to DLM on climbing behavior of *D. melanogaster*.

Climbing behavior of *D. melanogaster* adults after exposure to 0.59 µM DLM for 24 hours was determined by scoring the average distance (cm) climbed by the flies in 8 s. The total number of survived flies represents the sum of three independent experiments. The results are presented as mean ± SEM. Data were analyzed by a two-tailed Student's *t*-test. **P ≤ 0.005 significant differences between control and DLM-fed flies.

3.2.3 Effect of DLM on the AChE Activity

The AChE was measured. Although no significant difference was detected after 24 hrs, however, the AChE activity was inhibited by 14.03% in flies exposed to DLM. As shown in (Figure 18).

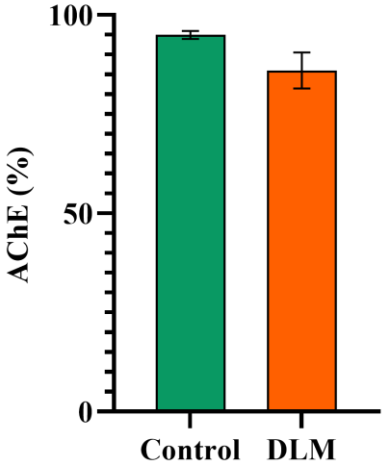

Figure 18: Acetylcholinesterase activity in flies exposed to DLM.

The AchE activity was measured after flies were exposed to DLM 0.59 µM for 24 h. Data were analyzed by a two-tailed Student's *t*-test. Results are expressed as a percentage of control (mean ± SEM); P ≥ 0.05 indicates no significant differences compared to the DLM group.

3.2.4 Effects of DLM on Gene Expression Profile of Dopaminergic System

Male flies exposed to 0.59 µM DLM for 24 hours showed increases in mRNA gene expression of all evaluated genes as compared to control flies (Figure 19) and (Figure 20). Statistical analysis revealed a significant upregulation effect of DLM on *dat* with 75.85% (p = 0.026) (Figure 18), *dop1r1* 85.20% (p = 0.0002), *dop2r* 67.06% (p = 0.0018), and *dopecr* 64.16% (p = 0.0024) (Figure 20). Although there was no statistically significant difference among the other genes, however, DLM caused upregulation in all tested genes with 26.90% *ple*, 51.66% *ddc*, and 28.74% *aanat1* (Figure 19).

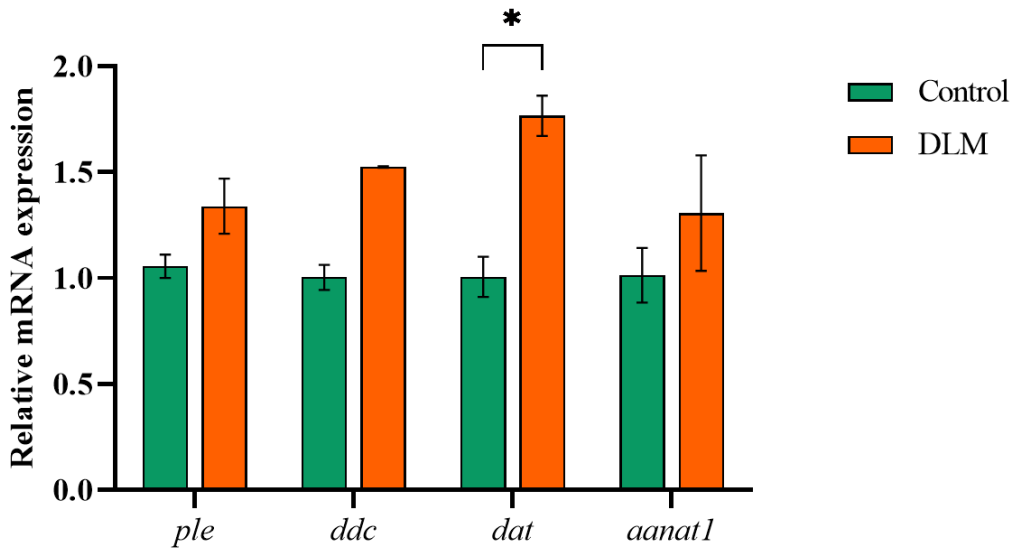

Figure 19: RT-qPCR gene expression of *ple*, *ddc*, *dat*, and *aanat1* in male flies exposed to 0.59 µM DLM for 24 hours.

Data represent mean fold change ± SEM relative to control flies. *P < 0.05 significant differences between control and DLM-fed flies. Data were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test.

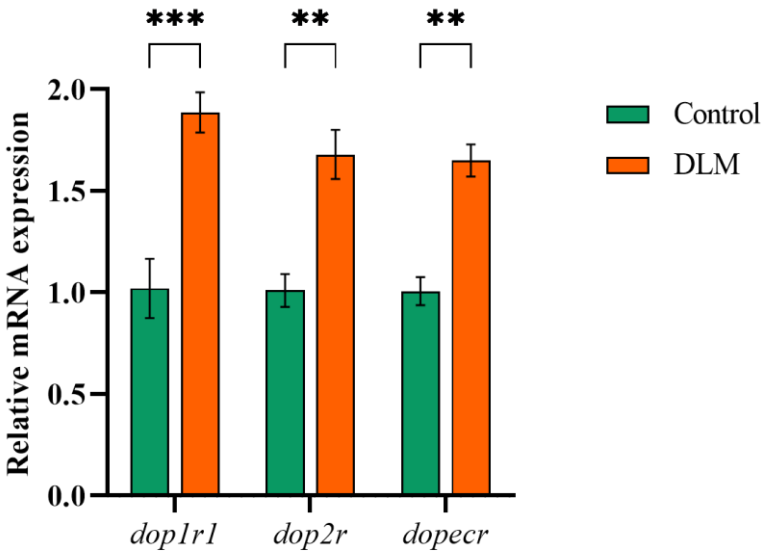

Figure 20: RT-qPCR gene expression of *dop1r1*, *dop2r*, and *dopecr* in male flies exposed to 0.59 µM DLM for 24 hours.

Data represent mean fold change ± SEM relative to control flies. **P ≤ 0.005, and ***P ≤ 0.0005 significant differences between control and DLM-fed flies. Data were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test.

3.3 DLM and Neuroprotective Agents

3.3.1 Effect of DLM and Neuroprotective Agents on the Survival of *D. Melanogaster*

Exposure of male adult flies to 0.59 µM of DLM for 72 hours resulted in a significant decrease in the percentage of survived flies 56.67% compared to the control 100% ($P < 0.0001$) (Figure 21). Co-exposure of flies to DLM and 250 µM FA, 25 µM TQ, and their combination significantly improved fly survival with 75.80%, 79%, and 74.40%, respectively (Figure 20). Administering FA, TQ, and their combination did not significantly affect the survival of the flies with 97.19%, 99.44, and 100.00%, respectively (Figure 21).

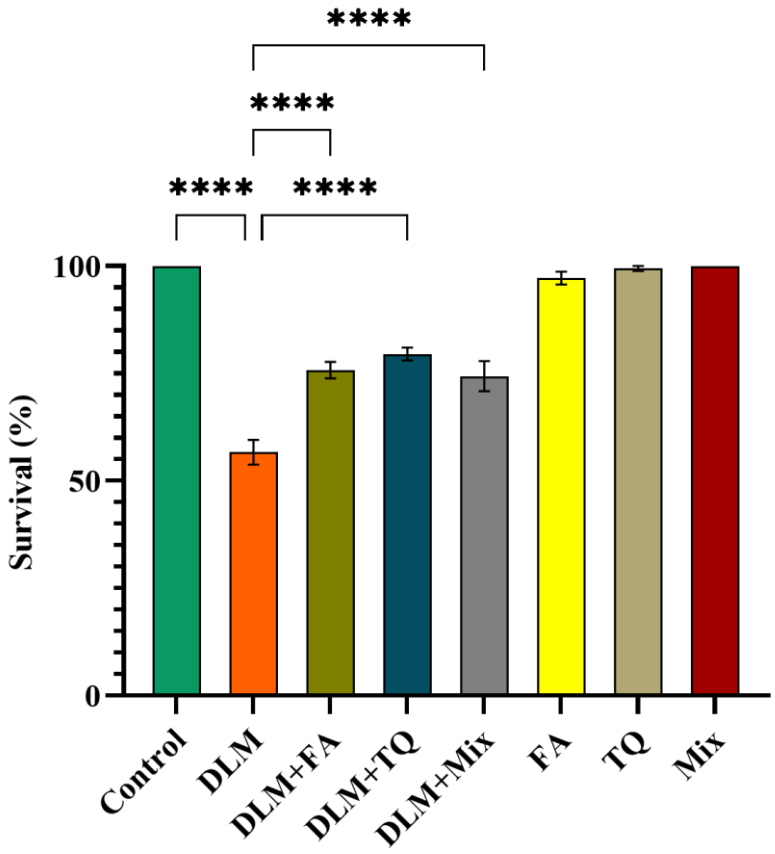

Figure 21: Effect of exposure to 0.59 DLM, 250 µM FA, 25 µM TQ, and their combination on the survival of wild-type flies.

The mortality was scored after 72 hours. The total number of flies (120 per group) represents the sum of three independent experiments. The results are represented as mean ± SEM and expressed as the average survival percentage of the flies, Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P ≤ 0.0001.

3.3.2 Locomotor Performance of *D. Melanogaster* Exposed to DLM & Neuroprotective Agents

Flies exposed to 0.59 µM DLM for 72 hours displayed a robust defect in reaching the top of the apparatus as compared to control flies indicating locomotor dysfunction. The average distance climbed by the control flies was 12.73 cm compared to 1.22 cm in DLM treated flies during 8 secs ($P < 0.0001$) (Figure 22). Co-exposure of flies to 250 µM FA, 25 µM TQ, and their combination prevented locomotor impairment induced by the DLM (Figure 22). Flies exposed to DLM+ FA, DLM+ TQ,

and DLM+ Mix climbed higher distances (3.48 (P = 0.0049), 3.27 (P = 0.0043), and 2.70 cm (P = 0.0425), respectively) than flies exposed to DLM alone (Figure 22). Administering FA, TQ, and their combination did not significantly affect the locomotor performance of flies with 12.35, 12.69, and 12.07 cm climbing distance, respectively (Figure 22).

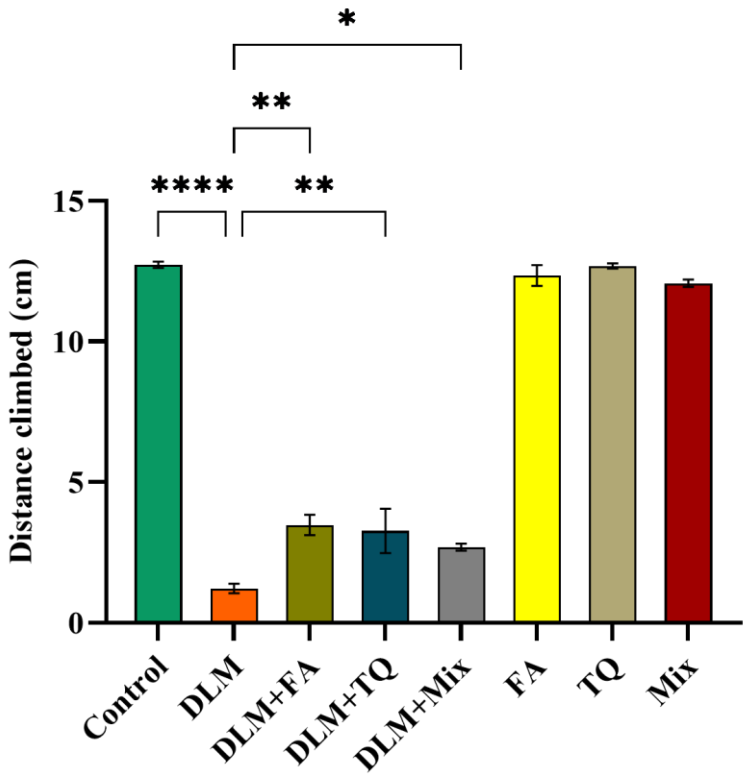

Figure 22: Effect of 72 hours exposure to DLM and neuroprotective agents on climbing behavior of *D. melanogaster*.

Climbing behavior was determined by scoring the average distance (cm) climbed by the flies in 8 s. The total number of survived flies represents the sum of three independent experiments. The results are presented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. *P < 0.05 **P ≤ 0.005, ***P ≤ 0.0001 significant differences between control and treatment.

3.3.3 Effect of DLM & Neuroprotective Agents on the AChE Activity

The AchE activity was significantly inhibited (p = 0.032) in flies exposed to DLM for 72 hours (48.30% decrease in AchE). As shown in (Figure 23), the co-exposure to the

different neuroprotective agents did not significantly restore the enzyme activity. However, FA alleviated the inhibition to 26.22%. Whereas, TQ did not show any improvement in the AChE activity, yet, the combined effect of FA, and TQ alleviated the inhibition to 25.04%. Neuroprotective agents did not show any significant effect on the AChE enzyme when administered alone.

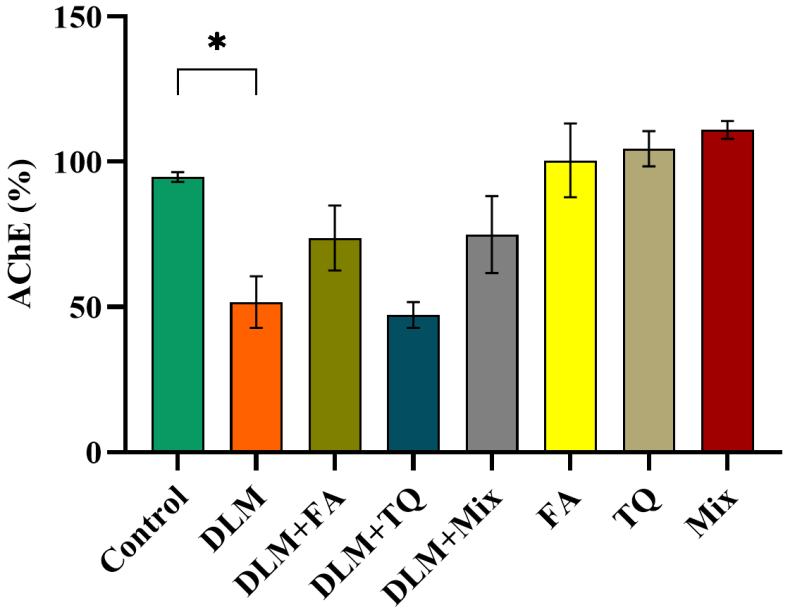

Figure 23: Acetylcholinesterase activity in flies exposed to DLM, individual neuroprotective agents, and their combinations.

The AchE activity was measured after flies were exposed to DLM 0.59 µM, TQ 25 µM, and FA 250 µM for 72 h. Results are expressed as a percentage of control (mean ± SEM); ∗p < 0.05 compared to the treatment group. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test.

Chapter 4: Discussion

Drosophila melanogaster is the closest invertebrate model organism to humans in terms of gene sequence similarity/conservation (Bier, 2005). It is well known for its high sensitivity to toxic substances and is considered a bioindicator for the detection of contaminants and for assessing the biological effects of pharmacological agents. In fact, *Drosophila* has been recommended by the European Centre for the Validation of Alternative Methods (ECVAM) for promoting the 3Rs (reduction, refinement, and replacement) of laboratory animals in toxicity research (Benford et al., 2000). *Drosophila melanogaster* has been successfully used to study the mechanisms underlying the pathophysiology of many neurological and non-neurological diseases in humans (Nichols, 2006; Beckingham et al., 2005).

Many studies have been conducted to investigate the effects of pesticides on the dopaminergic neuronal pathways, concerning the relationship between pesticide exposure and PD (Lockwood, 2000; Dick, 2006). Therefore, in this study, we chose *D. melanogaster* as an organism to test the neurotoxicity induced by insecticides and the potential neuroprotective effect of two naturally occurring compounds.

4.1 CPF Exposure for 24 Hours

Exposure to CPF has several harmful effects on human and animal health. A growing number of studies have linked an increased risk of PD with exposure to insecticides, particularly CPF (Freire & Koifman, 2012). Chlorpyrifos is one of the pesticides that is associated with increased expression of α-Synuclein, a protein that plays a crucial role in PD, in cell line model of dopaminergic neurons (Anderson et al., 2020). Furthermore, in rats treated with CPF, a significant reduction in dopaminergic

neurons was demonstrated, suggesting that CPF exposure may induce dopaminergic neuronal injury (Zhang et al., 2015). In the present study, CPF exposure for 24 hours using a concentration lower than the LC50 resulted in a significant decrease in flies' survival and the survivors exhibited significant locomotor deficits with a concomitant decrease in AChE activity. Our results are consistent with previous studies where *Drosophila* flies was exposed to almost the same concentration of CPF for 24 hours (Rodrigues et al., 2019) and another study where a lower concentration for 48 hours nearly gave similar results (Gomes et al., 2020). Acetylcholinesterase is known to hydrolyze ACh, a neurotransmitter that plays a central role in the regulation of motor function and locomotion (Day et al., 1991). Chlorpyrifos induces neurotoxicity via inhibition of AChE. Therefore, the decrease in AChE activity observed in this study could hinder normal neurotransmission and thus be associated with impaired climbing activity due to poor coordination between nervous and muscular junctions following CPF exposure (Adedara et al., 2015).

Although AChE, inhibition is the main effect of exposure to organophosphate insecticides. However, inhibition of AChE cannot fully explain the neurotoxicity of CPF. Studies show that CPF neurotoxicity persists even after cholinergic neurons have recovered and the effects appear not only in cholinergic-enriched regions but also in some other regions, such as the cerebellum (Moreno et al., 2008). Both *in vitro* and *in vivo* studies show that exposure to CPF can also alter catecholamine neurotransmitters such as DA (Slotkin et al., 2002; Chen et al., 2011). Only few studies have focused on the mechanism of action of organophosphate insecticides on DA metabolism (Ruan et al., 2006; Eells & Brown 2009). Most of these studies concluded that treatment with organophosphate insecticides could increase DA turnover (DOPAC / DA) with or without changes in DA levels. *In vitro* studies showed that CPF could cause a

significant increase in the gene expression of COMT, one of the main metabolic enzymes of DA (Slotkin & Seidler 2009). Another *In vitro* study revealed that MAO activity was decreased following incubation with CPF (Xu et al., 2012). These results suggest that CPF may interfere with the dopaminergic pathway.

The present study investigated the influence of CPF on DA-related genes in *D. melanogaster*. Several genes involved in DA dynamics (synthesis, secretion, transport) are conserved between *Drosophila* and mammals. Despite these similarities, no direct orthologs of MAO and COMT genes. Instead, *aanat1* is the enzyme responsible for DA metabolism in *Drosophila* (Yamamoto & Seto, 2014). *Drosophila* exposed to CPF for 24 hours showed no statistically significant differences between the control and the treatment groups among all tested genes. Nevertheless, we observed interesting trends in *ple* mRNA levels. Flies exposed to 2 µM CPF showed a reduction in *ple* mRNA levels as compared to control flies. While an increase in *ddc*, *dat*, *dop1r1*, *dop2r*, *dopecr*, and *aanat1* expression in males exposed to CPF was observed when compared with the control groups. The reason why there was no significant difference might be because the duration of exposure was not long enough to cause severe disturbance of the dopaminergic system. Exposure for a longer period is expected to significantly affect the tested genes as was shown in previous studies where the expression of TH had a remarkable decrease in CPF treated rats when administered for a long time. These changes in dopaminergic genes were accompanied by a significantly decreased motor activity. (Zhang et al., 2011; Sheikh & Sheikh, 2020).

Tyrosine hydroxylase is an important mediator concerning PD as a marker for dopaminergic neurons. Despite, CPF was shown previously to induce significant locomotor deficits in *D. melanogaster*, however, this defect was linked to the CPF main mode of action as an AChE inhibitor, in addition to the oxidative stress that can

be caused during organophosphates poisoning (Rodrigues et al., 2019; Gomes et al., 2020). The locomotor damage caused by CPF has been reported in several species, including aquatic organisms and rodents (Kavitha & Rao, 2008; Tilton et al., 2011; Zhang et al., 2011). Although, the effect of CPF on the TH expression in *D. melanogaster* has never been studied. However, it was reported that CPF caused a reduction in TH expression in rats which was accompanied by locomotion impairment (Zhang et al., 2011; Sheikh & Sheikh, 2020). Chlorpyrifos caused impairment in locomotor activity and reduction in *ple*, which is the rate-limiting step in the dopamine synthesis pathway in *D. melanogaster*, indicating that the toxicity of CPF is not confined only to the cholinergic system, but also it exerts dopaminergic system toxicity. Thus, our findings support previous studies that imply that CPF is a potent neurotoxin at concentrations lower than those that cause fatal inhibition of cholinesterase (Adedara et al., 2015).

Finally, it can be concluded that 24 hours exposure to CPF was able to disturb dopaminergic genes which may indicate other toxic effects of CPF other than the inhibition of AChE, and strongly support that it can be a risk factor in inducing PD because CPF caused a significant reduction of AChE associated with a significant motor deficit with a slight reduction in *ple* expression. However, further studies such as analyzing the gene expression after a long time of exposure and measuring direct DA content of the brain and how this can be linked to the behavioral deficits in *D. melanogaster* are needed.

4.2 New Feeding Device

In this study, a new simple feeding device was presented to be used in *Drosophila* toxicity studies. In many studies, changing the food is needed daily such

as in the case of neuroprotective agents and/or photosensitive chemicals. Thus, this device enables the dispensing of toxic molecules to flies while feeding on a sucrose solution. One major advantage of this device is that changing the toxic molecule or neuroprotective agent does not require anesthetizing the flies because the soaked cotton bud is attached to the foam plug, which can be readily replaced with a fresh one after tapping down the flies gently. Using the conventional cotton system has some drawbacks such as (1) using the same cotton for more than one day poses a high risk of fungal growth on the wet cotton; (2) it is not easy to replace the cotton for each treatment because flies will escape from the vial , therefore, losing the experimental samples; and (3) increasing the risk of flies being stuck in the sugar as there is a possibility of sugar dripping if the cotton got saturated.

The mortality and impaired locomotor performance of the *Drosophila* after exposure to CPF indicated that fruit flies were appropriately fed using this method.

4.3 Comparison Between Different Negative Geotaxis Methodologies

The most common negative geotaxis method is a relative measurement by calculating the number of flies passing a line marked at a certain height (8 cm for example) in the test vial. Although, this method is less time-consuming, nevertheless, it is less accurate especially with a treatment that can cause severe impairment in the locomotor activity. In such a case, biased results and conclusions may be obtained as we might have no or very little number of flies that will be able to reach or pass the predefined distance. On the contrary, calculating the average distance covered by all flies individually in the test vial is considered as an absolute measurement, that measures the average distance climbed by all flies and not just the ones that pass the line. This method is more accurate and more sensitive, especially when detection of

severe damage is required. Although other methods use the same approach manually by measuring the distance covered by each fly against a ruler captured inside the image (Barone & Bohmann, 2013), or using a graduate cylinder (Nichols et al., 2012; Madabattula et al., 2015), the measurements are not as accurate as of the method used in the current study which uses an image processing software because these methods are more prone to human errors. Moreover, they are more time-consuming. Although, there are other methods that use software the captures the position of each fly in the image or video to provide coordinates are either not free or not user-friendly. For example, the software Free Climber requires installing an Anaconda-based virtual environment as well as Python 3 virtual environment to be able to use the software (Spierer et al., 2021). Therefore, the current method (the modified RING assay) is very accurate, user-friendly, and above all, it is free of charge. The major advantages of the new method: (1) it is an absolute method by taking into consideration all flies and not just the ones that pass the line (6 or 8 cm), (2) the measurement of each fly distance is done very precisely by the software based on the number of pixels on the image. Unlike other methods in which the distance is nearly an estimation that is made based on a ruler present in the image, (3) the new method uses free software (PAST 4), which can be downloaded and used immediately unlike other methods that use proprietary software, which is not available for many researchers, and (4) the new method requires fewer replications because it takes the distance data from all flies in the experiment (entire fly population), unlike the methods in which data is taken only from the flies that pass the line (6 or 8 cm) and thus data comes from a sample of the fly population. Therefore, the new method saves a lot of time while being able to reveal the differences in the fly's behavior. In short, in this study we compared three methods of negative geotaxis assay and suggested that the modified RING assay was the most accurate and

reliable method for measuring the negative geotactic behavior of *D. melanogaster*. Especially, when severe damage is induced this method will be able to detect the climbing differences.

4.4 DLM Exposure for 24 Hours

Humans are exposed to DLM via different routes, either direct exposure to the vapors, epidermal contact, or ingestion of contaminated food. Acute and chronic exposure to DLM leads to the pathophysiology of neurodegenerative disorders like PD, AD, and learning disabilities. In this work, we investigated the molecular mechanisms involved in the neurotoxic actions of DLM after 24 hours of exposure in *D. melanogaster*. Exposing fruit flies to DLM for 24hours caused a significant reduction in the survival rate of flies. The surviving flies exhibited a significant reduction in the climbing abilities of the treated flies. In rats, similar observations were reported upon exposure to DLM where exposure to DLM resulted in decreased locomotor activity (Lazarini et al., 2001; Johri et al., 2006).

The effect of DLM on AChE activity was not significant. However, it exhibited a low level of inhibition explained by the short time exposure as well as that AChE is not the main mechanism of action of DLM like in case of OPs. The activity of AChE was significantly decreased in DLM-exposed rats when compared to the control group in previous studies (Mani et al., 2014; Khan et al., 2018). Again, the differences between our findings and the previous studies can be explained by the length of the exposure time. In particular, research has suggested a possible role for voltage-gated calcium channels, chloride channels, GABA receptors, in modulating neurotransmitter release, especially ACh, DA, and serotonin, in the acute and chronic manifestation of pyrethroid pesticides induced neurotoxicity (Soderlund et al., 2002). It was shown that

the content of DA in the striatum was significantly decreased in rats by DLM exposure (Liu et al., 2006; Tayebati et al., 2009).

The same DA-related genes that were tested after CPF exposure for 24 hours were also tested after DLM exposure for 24 hours. Deltamethrin altered the expression of DA-related genes. We observed significant upregulation of DA receptors with the highest upregulation observed in *dop1r1* followed by *dop2r, dopecr,* and the DA-transporter gene (*dat*). Although the other genes were not significantly altered, however, the trend of upregulation was present. Two possibilities could explain these upregulations: (1) the oxidative stress induced by DLM caused all genes to be upregulated as a rapid response; (2) Deltamethrin caused depletion of DA content in the flies and activated the negative feedback mechanism in which genes responsible for DA biosynthesis (*ple*, and *ddc*) were upregulated to compensate the loss of DA and DA- receptors (*dop1r1*, *dop2r*, and *dopecr*) were upregulated as a response of increase *dat* expression. It should be noted that the *dat* upregulation is documented as a sign of pyrethroid exposure and toxicity. Additionally, the gene responsible for DA metabolism (*aanat1*) was upregulated consolidating the DA loss hypothesis. One experiment showed that in DAT overexpressing mice, the levels of D1 and D2 receptors were significantly increased, suggesting that changes in DA-receptor found in DLM-exposed mice are the result of upregulation in response to an increase in the number of DAT and, subsequently, to a decrease in extracellular levels of DA (Richardson et al., 2015).

Consistent with our findings, a study reported that developmental DLM exposure increased striatal D1 levels in male mice (Richardson et al., 2015). On the contrary, DLM exposure resulted in decreased transcript levels of the *drd1*, increased levels of TH, and was associated with decreased *drd2a* transcripts in larval fish,

exposed developmentally to DLM. Developmental exposure of zebrafish to DLM significantly increased larval swim activity. (Kung et al., 2015). Several studies have reported a reduction in mRNA of TH in cell lines (Liu et al., 2006) and other vertebrate model organisms (Liu et al., 2006; Tayebati et al., 2006; Kung et al., 2015). The differences between our results and the previously mentioned studies might be due to the differential responses of developing and adult organisms to toxicant exposure as well as due to different organisms and the time of exposure. However, both studies indicate the potential for DLM to modulate TH expression, thereby altering DA biosynthesis. It was shown that exposure to the lowest concentration of DLM resulted in increased TH expression immediately after exposure. On the other hand, repeated exposure to DLM has been shown to decrease TH mRNA and protein expression in adult male rats (Liu et al., 2006).

Although the D1 and D2 auto-receptors have opposite effects on cAMP signal transduction, they act synergistically to modulate locomotor activity (Robertson, 1992). A change in D1 receptor signal transduction often leads to corresponding changes in D2 receptor-mediated responses; D1 regulates the sensitivity of D2 (Hasbi et al., 2011). Therefore, it is plausible that a sustained increase in *dop1r1* transcripts could promote a subsequent upregulation of *dop2r* at the transcriptional level, which we observed in our results.

4.5 DLM & Neuroprotective Agents Exposure for 72 Hours

The lipophilic nature of pyrethroids has quick and easy access to the tissues, including the central nervous system (CNS). Thus, even very small doses can produce significant biological and pathophysiological effects. Recently, many studies have reported that exposure to pyrethroid pesticides can cause mild to severe

neurophysiological and neurological behavioral disorders in humans (Mani et al., 2017). Exposure to pyrethroid pesticides has been found to affect neurological performance, neurotransmitter systems in humans, and experimental models (Wolansky & Harrill, 2008). Exposure to DLM also causes a decrease in the frequency of locomotion in parallel with an increase in immobility, suggesting a motor deficiency related to the dopaminergic blocking function. During subsequent exposure to pyrethroid pesticides, changes in motor activity were reported (Lazarini et al., 2001; Wolansky et al., 2008). In our work exposing *Drosophila* to DLM for 24 hours did not cause high mortality. We found that 80 % of flies were able to survive the used concentration. Therefore, we kept the flies on the same concertation for 72 hours. After 72 hours the mortality increased to double compared to the 24 hours. Fruit flies exposed to DLM developed severe climbing defects. The flies were not able to coordinate the movement of their legs and they were unable of climbing the tubes' walls. In addition, exposing the flies to DLM caused significant inhibition of AChE activity after 72 hours. This significant inhibition was not observed at 24 hours. Exposure for a long time caused more mortality and more damage in the nervous systems as is explained by the severely affected locomotor performance and AChE activity. It was shown previously that DLM caused inhibition of the AChE activity in rats which was explained by the ability of DLM to lower the ACh binding space at the aromatic, hydrophobic surface of AChE, most likely due to its lipophilic nature (Mohammadi et al., 2019).

In recent years, scientific studies have drawn attention to the beneficial health effects of plant-based foods and their active ingredients. Free radical scavenging appears to play an important role in the antioxidant activity of natural products, from this the role of natural compounds had recently received considerable attention as a

dietary antioxidant. The neuroprotective effect of FA and TQ has been reported in different experimental studies (Ojha et al., 2015; Farkhondeh et al., 2018). Hence, the present study was designed to test the neuroprotective effect of FA, TQ, or their combination against DLM-induced neurotoxic damage, behavioral and biochemical changes in the *D. melanogaster*. Co-administering DLM with neuroprotective agents was able to improve the survival of flies significantly. Neuroprotective agents were also able to improve the negative geotaxis of *Drosophila* significantly. Combining FA and TQ did not add any extra protection on the mortality as well as the negative geotaxis indicating that there was neither synergistic nor additive effect.

Ferulic acid co-treatment altered the levels of AChE to the normal level. However, the change was not significant, and this might be due to the concentration that was used. More experiments to optimize the concentration that can give maximum beneficial effects with minimum side effects are needed. On the other hand, TQ at 25 µM did not improve the enzyme activity. Despite this, it was shown in our study that it protected against DLM-induced mortality and climbing deficits. Yet, its protective effect might be due to another mechanism of action than affecting the AChE activity. Combining FA and TQ did not further improve the enzyme activity indicating that the neuroprotective effect was due to the individual effect of FA alone. There were no significant differences between DMSO and DLM co-administered with FA and the combined FA+TQ. This means that FA was able to restore the enzyme activity to the levels of the control. Because TQ did not affect the FA neuroprotective effect on AChE which means that it doesn't interfere with the mechanism of protection of the FA.

Drosophila exposed to pure neuroprotective agents without DLM did not alter any of the parameters tested indicating the safety of the chosen concentrations. However, FA, TQ, and their combination slightly elevated the AChE activity even

more than the control and that is why it was shown in (Figure 22) that FA, TQ, and the Mix had higher than 100% enzyme activity.

Studies reported that FA significantly increased the intensity of TH fibers, suggesting a neuroprotective effect mediated by FA. Therefore, suggesting that treatment with FA is beneficial to DA neurons, which protected them from rotenone-induced toxicity (Ojha et al., 2015). One more study showed that FA was able to reverse the toxic effect of cadmium-induced brain damage on cholinesterase activities in rats (Adefegha et al., 2016). In addition, it was shown in a previous study that TQ rescued the levels of AChE that was inhibited by malathion, an OP insecticide (Abdel-Daim et al., 2020). This result is inconsistent with our finding because in our study TQ did not reverse the inhibitory effect of DLM on the AChE enzyme. This might be due to the dose that we used was not sufficient to reverse the inhibition. Another reason could be due to the different mechanisms between OPs and DLM on the AChE.

Chapter 5: Conclusion

In conclusion, we introduced a new simple feeding device for *D. melanogaster*, which can be assembled and used in any laboratory. In addition, the findings of the present study suggest that the three negative geotaxis assays that were tested in this study are suitable for detecting changes in climbing behavior of the *D. melanogaster* adults. However, we recommend the modified RING assay for a more accurate and reliable negative geotaxis measurement. Furthermore, this study has shown that the CPF at 2 µM and DLM at 0.59 µM were able to cause severe damage in adult *D. melanogaster* treated for 24 hours, including increased mortality (less than 50%), locomotor deficits, inhibition of AChE, and disturbing dopaminergic pathways in terms of gene expression. Two main systems responsible for coordinative movement were disturbed using two different classes of insecticides. This study provides evidence that pesticides can exsert several mechanisms by which they can cause neurotoxic effects and these mechanisms are not limited to one system affected at a time. Additionally, this study clarifies that DLM exposure for different periods resulted in varying degrees of severity on the neurobehavioral parameters and AChE activity. This study reported for the first time the neuroprotective action of FA and TQ against the DLM-induced neurotoxicity. The FA alleviated the inhibition of AChE by DLM thus, attributing a neuroprotective potential to this natural compound. Therefore, FA may be considered as a promising source of potential therapeutic agents for the treatment of DLM intoxications. Together, the new cotton swab feeding device, the modified negative geotaxis assay, and the 24 hours of exposure to either CPF, or DLM at the provided concentrations present a complete, simple, and fast system to study neurodegenerative damage and neuroprotective agents using *D. melanogaster* as a model organism. These pesticides were able to disturb two neurological systems at 24

hours so it is expected that longer exposure will cause persistent damage which could contribute to the development of sporadic PD. Furthermore, we recommend testing the neuroprotection effect of FA and TQ on the dopaminergic pathways.

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